THE REGULATION OF TYPE I COLLAGEN GENE EXPRESSION IN STROMAL FIBROBLAST BY BREAST TUMOUR CELLS.

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ABBREVIATIONS

ADAM	A disintegrin and metalloprotease
ATCC	American type culture collection
ATP	Adenosine triphosphate
α-SMA	Alpha smooth muscle actin
β-gal	Beta-galactosidase
BSA	Bovine serum albumin
CAF	Carcinoma-associated fibroblast
cAMP	Cyclic adenosine monophosphate
CBP	CCAAT binding protein
cDNA	complementary DNA
CMV	Cytomegalovirus
CTGF	Connective tissue growth factor
DAMP	Damage-associated molecular pattern
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxyribonucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated protein kinase
FACS	Fluorescence-activated cell sorting

FAK	Focal adhesion kinase
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HGF	Hepatocyte growth factor
HSPG	Heparin-sulfate proteoglycans
ICAM	Intercellular adhesion molecule
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IL	Interleukin
IRF	Interferon regulatory factor
ISGF	Interferon stimulated gene factor
ISRE	Interferon stimulated response element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MCP1	Monocyte chemoattractant protein 1
MEK	Mitogen-activated protein kinase kinase
MIP1	Macrophage inflammatory protein 1
MMP	Matrix metalloproteinase
MOPS	3-[N-Morpholino] propane sulphonic acid
mRNA	messenger RNA
NFκβ	Nuclear factor kappa-light-chain-enhancer of activated B cells.

NLR	NOD-like receptors
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PRR	Pattern recognition receptor
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
Smad	Sons of mothers against decapentaplegic
SPARC	Secreted protein, acidic and rich in cysteine
Stat	Signal transducers and activators of transcription
TAM	Tumour-associated macrophage
TCA	Trichloroacetic acid
TGFβ	Tumour growth factor beta
TNFα	Tumour necrosis factor alpha
TLR	Toll-like receptor
Tris	Tris(hydroxymethyl)aminomethane
TSP-1	Thrombospondin-1
TYK	Tyrosine kinase
VCAM	Vascular cell adhesion molecule

ABSTRACT

Recent studies have revealed that interactions between tumour cells and the surrounding stroma play an important role in facilitating tumour growth and invasion. Stromal fibroblasts produce most of the extracellular matrix (ECM) components found in the stroma, including type I collagen. Previous *in vivo* studies in our laboratory have shown that type I collagen mRNA levels are decreased in stage II and III breast tumour tissue compared to adjacent normal tissue. Since type I collagen forms the main constituent of the ECM, the role of tumour cells in regulating the synthesis of type I collagen in neighbouring fibroblasts could have important implications for tumour invasion. The aim of this study was therefore to investigate the mechanisms involved in breast tumour-mediated regulation of type I collagen gene expression in neighbouring fibroblasts.

Results of co-culture experiments showed that MDA-MB-231 breast tumour cells negatively regulated type I collagen gene expression when in close contact with either CCD-1068SK breast skin or WI38 lung fibroblasts. Down-regulation of both the COL1A1 and COL1A2 genes was observed, with specific involvement of the -375/+54 region of the COL1A2 promoter. Since non-tumourigenic MCF12A epithelial cells did not affect type I collagen gene expression in co-cultured fibroblasts, the negative regulatory effect on type I collagen expression appeared to be tumour-specific. Further ELISA analysis of direct co-culture medium revealed that well-known negative regulators of type I collagen such as TNFα, IFNγ and IL-1, were not secreted during tumour/fibroblast co-cultures. However, the expression of IFNα and IFNβ was up-regulated in fibroblast/tumour co-cultures. Type I IFNs activate JAK/STAT signalling and further investigation of this pathway revealed that Stat1 mRNA and protein levels were significantly up-regulated in both fibroblasts and tumour cells after direct co-culture. Increased Stat1 expression and activation in CCD-1068SK fibroblasts was shown to occur as a result of a factor secreted during CCD-1068SK/MDA-MB-231 direct co-cultures, which we suggest is a type I IFN. However, inhibition of JAK/Stat signalling did not reverse the negative regulatory effect of tumour cells on type I collagen gene expression.

Gene expression analysis performed on co-cultured CCD-1068SK fibroblasts showed that MMP-1 was overexpressed, while CCN2 gene expression was down-regulated as a result of close contact with tumour cells. Inhibiting CCN2 gene expression in fibroblasts led to a significant decrease in type I collagen gene expression levels in fibroblasts, suggesting a role for this matricellular protein in tumour-mediated regulation of type I collagen. Increased expression of Smad7 observed in co-cultured fibroblasts was shown to play a role in tumour-mediated suppression of CCN2 gene expression. However, inhibition of Smad7 gene expression in fibroblasts did not reverse the negative regulatory effect of tumour cells on type I collagen gene expression.

The results obtained in this study suggest that tumour cells are able to down-regulate the expression of profibrotic genes such as type I collagen and CCN2, while up-regulating matrix-degrading MMP gene expression, in fibroblasts when in close contact with these cells. This type of close contact between tumour cells and fibroblasts is only possible in the later stages of breast cancer progression, when the basement membrane separating these two cell types has been degraded, and the resulting decrease in fibroblast-mediated production of the surrounding extracellular matrix could facilitate further tumour invasion and metastasis.

CHAPTER ONE

INTRODUCTION

1.1 Preamble

Epithelial cell cancers are believed to occur as a result of acquired or inherited genetic defects in the DNA of these cells. Such mutations cause the cells to proliferate at a higher rate than normal and in due course uncontrollably, leading to the development of a carcinoma. While some carcinomas remain benign others invade the surrounding stroma and may subsequently metastasize to other parts of the body, resulting in the major cause of cancer deaths.

Cancer studies have previously focused on how and why epithelial cell mutations occur, but recent evidence has shown that the stroma surrounding the epithelial cells plays a crucial role in both the promotion and progression of carcinomas. The stroma consists of the extracellular matrix (ECM) and a number of different cell types, including fibroblasts, pericytes, immune and inflammatory cells (Weigelt & Bissell 2008). The stroma makes up 80% of normal human breast tissue which is essential for preserving normal epithelial structure and adhesion and serves as a barrier that impedes tumour development (Beacham & Cukierman 2005; Parmar & Cunha 2004). If tumourigenesis occurs, the carcinoma cells communicate with the surrounding stromal cells causing abnormal changes in stromal homeostasis which may then promote further tumour invasion and metastasis (Bierie & Moses 2006).

The focus of this chapter is to discuss previous studies performed on breast cancer and the role of the stroma in tumour initiation and development. More specifically, tumour/fibroblast interactions during different stages of tumour progression and the effect of these interactions on type I collagen gene expression will be addressed.

1.2 Breast cancer

Breast cancer is the most common cancer in women worldwide, with an estimated 1.38 million women diagnosed with breast cancer in 2008 alone (Ferlay et al. 2008). In South Africa, breast cancer is also the most commonly diagnosed cancer among women, with an estimated incidence rate of 41/100 000 women and a mortality rate of 20.7/100 000 women (Ferlay et al. 2008). Increased awareness and diagnosis at earlier stages of the disease have led to a decrease in the mortality rate in developed countries, but in developing countries breast cancer still remains the most common cause of cancer deaths in women. Once tumours have metastasized to secondary sites, the chances of survival are significantly decreased (Allinen et al. 2004). A better understanding of the molecular mechanisms involved in tumour invasion and metastasis are therefore essential for the development of improved tumour-targeted therapies.

The normal human mammary gland is composed of a branching ductal-lobular system. Fifteen to twenty lobes make up each lobule, and these are drained by collecting ducts that converge at the nipple. The lobules, known as terminal duct lobular units, consist of acini (alveoli) that function as the secretory units of the mammary gland during lactation (Weigelt & 2008). Two types of glandular epithelial cells make up the mammary ducts and acini; the continuous inner layer of luminal epithelium surrounds the hollow lumen while the outer myoepithelial cell layer is in direct contact with the basement membrane (figure 1.1 A) (Debnath & Brugge 2005; Kalluri & Zeisberg 2006). Myoepithelial cells express type IV collagen, laminin, smooth muscle actin, and oxytocin receptor and therefore play an important role in the formation of the basement membrane and lactation (Gudjonsson et al. 2002). The laminin-1 produced by these cells is essential for the control of luminal cell polarity and differentiation (Gomm et al. 1997; Gudjonsson et al. 2002). Myoepithelial cells are also believed to suppress breast cancer cell growth, invasion, and angiogenesis by expressing CD44 and proteinase inhibitors (Alpaugh et al. 2000; Barsky 2003; Xiao et al. 1999). The epithelial cells of the ducts and glands are separated from the surrounding stroma by the basement membrane, which makes up approximately 80% of breast tissue and consists mainly of varying amounts of fat, connective tissue, nerves, blood vessels and lymphatic vessels (Weigelt & Bissell 2008). This extracellular matrix is

essential for preserving normal epithelial structure and adhesion and serves as a barrier that impedes tumour development (Beacham & Cukierman 2005; Parmar & Cunha 2004). A number of different cell types are also found in the stroma; including fibroblasts, immune cells, endothelial cells, pericytes and inflammatory cells.

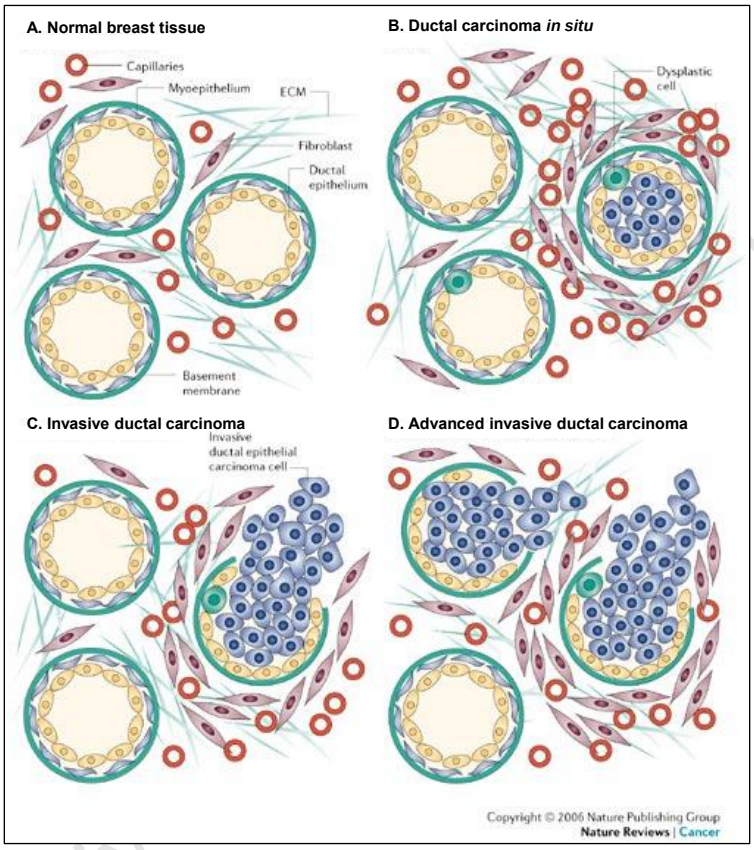

Figure 1.1 (A) In normal female breast tissue, two layers of glandular epithelial cells are separated from the surrounding stroma by the basement membrane. The stroma consists of extracellular matrix (ECM) components, as well as blood vessels and fibroblasts. (B) In non-invasive ductal carcinoma *in situ* (DCIS), transformed epithelial cells proliferate to fill the lumen. Fibroblasts are also recruited to the tumour site where they are activated and deposit ECM components such as type I collagen. (C) Progression to an invasive ductal carcinoma is characterized by basement membrane degradation, which allows the tumour cells to migrate into the surrounding stroma. Changes to the stroma include increased angiogenesis and the recruitment of immune and inflammatory cells. (D) During advanced stages of breast carcinoma, further dedifferentiation of the stroma occurs with the tumour cells forming irregular "nests and cords" as they invade the stroma. (Kalluri & Zeisberg, 2006).

Most genetic mutations occur in the luminal epithelial cells of the terminal duct lobular unit, while myoepithelial carcinomas are not very common and are mostly benign (Sternlicht et al. 1997; Gudjonsson et al. 2006). The early stages of tumour development, characterised by loss of luminal epithelial cell polarity, dedifferentiation and proliferation

of the abnormal cells, is known as non-invasive ductal carcinoma *in situ* (DCIS) (figure 1.1 B). The lumen may become filled with the transformed cells, but they remain within the duct and are separated from the surrounding stroma by the basement membrane. Further tumour progression is characterised by decreased structural organisation of the acinar structure, with displacement of the myoepithelial cells and degradation of the basement membrane. If the carcinoma cells migrate and invade the surrounding stroma, invasive ductal carcinoma is diagnosed, which makes up 70% of all breast cancers (figure 1.1 C) (Debnath & Brugge 2005; Wozniak & Keely 2005). Tumour development is often accompanied by a loss of myoepithelial cells, increased angiogenesis and a larger number of myofibroblasts and immune cells in the surrounding stroma (Gudjonsson et al. 2006; Shekhar et al. 2003; Weigelt & Bissell 2008). Carcinoma cells also communicate with the surrounding stromal cells causing abnormal changes to the extracellular matrix, with decreased structural differentiation often observed, especially at the invasive front of the tumour mass (Bierie & Moses 2006; van Kempen et al. 2005).

Tumours of the mammary gland are quite heterogeneous with regards to morphology, gene expression and clinical behaviours. Tumours can be classified into three different grades based on histological assessment (Lacroix et al. 2004); grade I tumours are low grade tumour and have quite well differentiated cells, grade III tumours are high-grade and display very poor differentiation, and grade II tumours fall between the other two categories. Further division of tumour into 18 subtypes is based on tumour cell growth rates and cytological traits (Kreike et al. 2007; Weigelt & Bissell 2008). However, these classification systems are very subjective and misclassification may occur (Weigelt et al. 2008).

The introduction of cDNA microarray technologies has allowed for classification of breast tumours into five subtypes based on gene expression patterns (Perou et al. 1999; Perou et al. 2000; Sorlie et al. 2001). Tumours were divided into two groups based on whether or not they expressed oestrogen receptor-α (ER). Oestrogen positive (ER+) tumours are derived from luminal epithelial cells and have been further divided into two subtypes; namely, luminal A and luminal B tumours. Oestrogen negative (ER-) tumours are divided

into HER2 positive, normal breast-like and basal-like subtypes, with the latter making up about 15 to 20% of breast cancers (Perou et al. 2000; Wienke et al. 2007).

Gene expression profiling of breast cancers has recently been used to develop diagnostic tests to profile clinical samples (Sotiriou & Pusztai 2009). Researchers from the Netherlands Cancer Institute developed a gene prognosis profile based on the expression of 70 genes (van't Veer et al. 2002; van de Vijver et al. 2002) which can be used to classify tumours into either a 'good' or 'poor' prognosis group. This 70 gene signature has recently been commercialised as the MammaPrint® (Agendia BV, Amsterdam, The Netherlands) and has been approved by the Food and Drug Administration (FDA) for clinical use (Sotiriou & Pusztai 2009). This gene signature has proven most useful in clinical assessment of intermediate grade tumours (e.g. intermediate expression of ER and grade II tumours) which could not be characterised further using conventional clinical and pathological assessment (Sotiriou et al. 2006; Nuyten & van de Vijver 2007; Sotiriou & Pusztai 2009). When compared with the Adjuvant! Online program (www.adjuvantonline.com), which uses conventional pathological features to classify tumours, the MammaPrint® gene signature also predicted the outcome more accurately (Sotiriou & Pusztai 2009). A number of other genome-based assays are now being developed, including Oncotype DX® (Genomic Health, Redwood City, California), H/I (AvariaDX, Carlsbad, California), Theros and MapQuant Dx (Marchionni et al. 2008; Sotiriou & Pusztai 2009). Although these tests have aided in predicting prognosis and clinical outcome for patients with ER+ tumours, they still classify all ER- tumours as high-risk (Sotiriou & Pusztai 2009; Straver et al. 2010). Since the worst prognosis is given for patients with ER- basal-like tumours (Kreike et al. 2007), further characterization of this group is needed to allow for improved prognosis and treatment.

1.3 Tumour-stromal interactions

For a number of years cancer has been regarded as a cell-autonomous process resulting from the accumulation of DNA mutations in a single somatic cell. However, more recent studies have shown that this "somatic mutation theory" is an oversimplified view of carcinogenesis and that tumour initiation and development is, in fact, a process that

involves continuous mutual interactions between tumour cells and their microenvironment (Sonnenschein & Soto 2008; Kenny et al. 2007). Initiation and progression of tumours to an invasive and metastatic state is therefore a combination of genetic and epigenetic changes as well as abnormal interactions with the surrounding stroma (Vargo-Gogola & Rosen 2007).

The stroma is made up of extracellular matrix components such as type I collagen and fibronectin as well as a number of different cell types including fibroblasts, endothelial cells, immune and inflammatory cells. During early stages of tumour formation, the tumour cells are separated from the surrounding stroma by the basement membrane which acts as a barrier to prevent further tumour growth (Beacham & Cukierman 2005). However, complex tumour-stromal interactions may result in changes to the stroma that facilitate breakdown of the basement membrane, allowing tumour cells to invade the surrounding ECM. Here, the tumour cells interact with both ECM components and stromal cells in a way that would not occur under normal, facilitating tumour invasion and metastasis (Bissell et al. 2002; Shekhar et al. 2003; Tlsty & Hein 2001; Beacham & Cukierman 2005; Parmar & Cunha 2004; Gaggioli 2008; Hu & Polyak 2008). Stromal fibroblasts are responsible for synthesizing and depositing most of the ECM components and, therefore, interactions between tumour cells and fibroblasts play an important role in determining how tumour cells are able to alter the ECM to facilitate tumour invasion.

1.3.1 Fibroblasts as key players in tissue remodelling.

Fibroblasts are mesenchymal cells found in the stroma of all epithelial organs in the body. They produce most of the components of the ECM, including collagens, glycosaminoglycans, reticular and elastic fibres, and glycoproteins. They also secrete ECM-degrading proteases such as matrix metalloproteinases (MMPs), and therefore play an important role in maintaining tissue homeostasis. Fibroblasts also communicate with their environment by producing these ECM components and by secreting soluble factors in response to external stimuli (Bierie & Moses 2006; Kalluri & Zeisberg 2006).

Fibroblasts play an essential role in organ development and in the maintenance of epithelial structure. Type IV collagen and laminin secreted by fibroblasts form important components of the basement membrane that separate the epithelial cells from the surrounding stroma (Kalluri & Zeisberg 2006). Kuperwasser et al. (2004) showed that epithelial cells only formed ductal, lobular and acinar structures that resembled those found in human mammary glands when they were co-injected with immortalized human breast fibroblasts into the cleared breast pads of NOD/SCID mice. However, no such structures formed when epithelial cells were injected on their own or with mouse fibroblasts. This study also highlights the species-specific role of fibroblasts in mammary gland differentiation.

Fibroblasts usually remain dormant and are sparsely distributed in adult tissue (Beacham & Cukierman 2005). Fibroblast activation usually only occurs in adult tissue during tissue injury or during disease conditions such as fibrosis, inflammatory diseases and cancer. The activated fibroblasts, also known as myofibroblasts, are characterised by high levels of α-smooth muscle actin (α-SMA), increased production of ECM components such as type I collagne, and an increased ability to contract tissue (Bierie & Moses 2006). The presence of active TGFβ and extra type III domain-A (EDA) fibronectin appear to be important for induction of the myofibroblast phenotype (Ronnov-Jessen et al. 1995; Shephard et al. 2004; Ding et al. 2008) and growth factors such as EGF (epidermal growth factor), PDGF (platelet-derived growth factor) and FGF-2 (fibroblast growth factor 2), as well as direct cell-cell communication with leukocytes through ICAM1 (intercellular-adhesion molecule 1) or VCAM1 (vascular-cell adhesion molecule 1) can also result in fibroblast activation (Kalluri & Zeisberg 2006). Little is known about the negative regulation of myofibroblast formation, but cytokines such as IFNγ (interferon gamma) and IL1 (interleukin 1) appear to be involved (Shephard et al. 2004; Tanaka 2003).

During wound healing myofibroblasts proliferate and migrate to the wounded area by binding to matrix components such as fibronectin, vitronectin and fibrin, via their integrin receptors (Ovington & Schultz 2004). Fibroblasts are able to chemotactically detect the gradient of growth factors, cytokines and chemokines released at the sight of injury and consequently pull themselves along the matrix fibrils in the direction of the wound site

(Ovington & Schultz 2004). The fibroblasts also secrete proteolytic enzymes such as matrix metalloproteases (MMPs) to facilitate their movement through the matrix and to assist in matrix remodelling (Eckes et al. 2000; Singer & Clark 1999). Fibroblasts usually only reach the wound site after the initial inflammatory phase (2 to 3 days), where they synthesize new connective tissue and promote wound closure by tissue contraction (Shephard et al. 2004; Ovington & Schultz 2004). After the wound has healed the myofibroblasts may either return to their resting phenotype or undergo apoptosis (Hinz et al. 2007; Eyden et al. 2009).

In disease conditions such as fibrosis and cancer, fibroblasts maintain an activated state without returning to the resting phase (Kalluri & Zeisberg 2006). However, recent literature suggests that the carcinoma-associated fibroblasts (CAFs) observed in tumour tissue may consist of activated fibroblasts together with other cell types (Ronnov-Jessen & Bissell 2008; Franco et al. 2010) such as smooth muscle cells and pericytes from the vasculature (Hinz et al. 2007) as well as fibrocytes and bone-marrow-derived mesenchymal stem cells (Ishii 2003; Forbes et al. 2004; Chauhan et al. 2003). Other studies suggest that epithelial cells that have undergone epithelial-to-mesenchymal (EMT) transition can also develop myofibroblast-like characteristics (Kim et al. 2006; Petersen 2003; Willis et al. 2005; Selman & Pardo 2006).

A better understanding of the definition of a myofibroblast will also assist researchers in determining the origin and functions of CAFs in tumours. Although myofibroblasts are characterised by the expression of α-SMA (alpha smooth muscle actin), this protein is also expressed by other cells such as pericytes, some endothelial cells, myoepithelial cells and epithelial cells undergoing EMT (Eyden 2009). Therefore, techniques such as immunohistochemistry are unable to distinguish these cell types from each other. Studies claiming that EMT-derived cells make up a part of the myofibroblast population have either used light microscopy techniques (Willis et al. 2005; Radisky et al. 2007) or have been unable to show certain characteristics of fully differentiated myofibroblast (Ng et al. 1999) such as a rough endoplasmic reticulum and the presence of fibronexuses (Eyden 2009). Electron microscopy has, however, proven to be useful for distinguishing

myofibroblast-specific features; for example, smooth-muscle cells and myofibroblasts both have myofilaments, but only the former has a lamina which can only be detected by means of electron microscopy. Pericytes can also be distinguished by the fact that they are poorly differentiated smooth-muscle cells and are usually found near endothelial cells.

Although the exact contribution of each cell type to the CAF population is still under debate (Rønnov-Jessen & Bissell 2008; McAnulty 2007), previous studies on CAFs suggest that this population is able to facilitate tumour development (Shimoda et al. 2010; Kunz-Schughart et al. 2001; Orimo 2005; Tyan et al. 2011). On the other hand, normal fibroblasts are believed to inhibit tumourigenesis, but their role in later stages of tumour invasion is still not well understood (Kalluri & Zeisberg 2006).

1.3.1.1 The role of fibroblasts in tumour initiation.

Whether stromal changes precede and facilitate tumourigenesis or rather occur in reaction to the tumour is still not clear, and it is possible that either may occur depending on the situation (Reilly & Van Dyke 2008). De Wever and Mareel (2003) suggest that tumour cells and the stroma may interact via an efferent or afferent pathway. In the efferent pathway tumourigenic cells may secrete and/or deposit soluble growth factors in the surrounding ECM, leading to a reactive stromal response which then facilitates further tumour development. In the afferent pathway reactive stromal cells such as myofibroblasts trigger changes in epithelial cells, leading to tumourigenesis.

A further alternative is that benign mutations present in epithelial cells rely on stromal changes to facilitate their conversion to a malignant state. Studies have shown that tumours are often unable to overcome the limitations imposed on them by the normal microenvironment unless the stroma itself is altered (Beacham & Cukierman 2005; De Wever & Mareel 2003; Kuperwasser 2004). Under normal conditions the stromal environment forms a barrier that prevents tumour initiation or progression, and fibroblasts in particular play an important role in regulating epithelial cell growth and differentiation (Beacham & Cukierman 2005; Kuperwasser et al. 2004). Non-malignant mammary

epithelial MCF10A cells that were co-cultured with normal primary mammary fibroblast in reconstituted basement membrane formed structures that resembled the terminal ductal-lobular units observed in normal breast tissue or early stage DCIS (Shekhar et al. 2001). Normal fibroblasts also reduced the proliferation of MCF10A cells and of the more progressively transformed cell line, MCF10AT (Sadlonova et al. 2005). However, normal fibroblasts may only be able to regulate tumour cell growth and proliferation up to a certain stage in the malignant transformation process, after which the tumour cells appear to be resistant to the fibroblast's cues (Mueller & Fusenig 2002).

1.3.1.2 The role of fibroblasts in tumour progression

Tumour development and progression is characterised by continuous interactions between the tumour cells and the surrounding stromal fibroblasts (Elenbaas & Weinberg 2001) and these interactions may vary depending on the stage of tumour progression. One of the factors that determine the type of interactions that occur is the distance between tumour cells and fibroblasts.

In the early stages, cancer cells are separated from the surrounding stroma by the basement membrane, as observed during the DCIS stage of breast cancer. This 'reactive stroma' resembles that found at the proliferative phase of wound healing, with increased numbers of activated fibroblasts, increased angiogenesis and the deposition of type I collagen and fibronectin (Zeisberg & Kalluri 2004; Rønnov-Jessen et al. 1996). These stromal changes imply that the tumour cells are able to communicate with fibroblasts through the basement membrane via secreted factors. Some tumours never progress past the DCIS stage, although the role of the tumour environment in either preventing or facilitating progression to the invasive stage is not well understood (Franco et al. 2010). Invasive ductal carcinomas are characterised by the degradation of the basement membrane, which allows tumour cells to invade the reactive stroma and come into close contact with the activated stromal fibroblasts (Zeisberg & Kalluri 2004; Tran-Thanh & Done 2010). These carcinoma-associated fibroblasts (CAFs) represent the most common cell type found in the stroma of invasive breast carcinomas and are responsible for the desmoplastic response,

which involves increased deposition of ECM components such as type I collagen, fibronectins, proteoglycans and glycosaminoglycans (Rønnov-Jessen et al. 1995; Egeblad et al. 2005). It is still not known whether this desmoplastic reaction is a wound-healing response to try and 'repair' and contain the tumour or if it actually facilitates tumour invasion (Zeisberg & Kalluri 2004).

A number of factors may be involved in cross-talk between CAFs and tumour cells, resulting in accelerated tumour growth and development. Tumour xenografts produced by ras-transformed human MCF-7 breast cancer cells co-injected with CAFs into nude mice were significantly larger than those produced by tumour cells mixed with normal fibroblasts (Orimo et al. 2005), with secretion of stromal cell-derived factor 1 (SDF-1) by CAFs shown to play an important role in this increased tumour growth as well as in angiogenesis. CAFs also secrete proteases such as matrix metalloproteases, cathepsins and plasminogen activators (Joyce & Pollard 2009) and production of MMP-1, MMP-2 and MMP-9 has been associated with invading and metastasizing tumours (Kunz-Schughart et al. 2001). Protease-mediated degradation of the ECM could facilitate tumour invasion by breaking adhesions between tumour cells and neighbouring cells or the matrix and allowing tumour cells to move freely through the degraded matrix (De Wever et al. 2008; Pietras & Ostman 2010). Growth factors embedded in the matrix are also released as a result of degradation, and these may interact with tumour and stromal cell surface receptors and facilitate further tumour invasion (Chung et al. 2005; Pietras & Ostman 2010).

The solid malignant tumour formed during the invasive stage usually consists of a necrotic centre surrounded by a peripheral region that consists of mixed tumour cells and stromal components. At the edges of this peripheral region lies the invasive front and this is separated from the adjacent uninvolved tissue by the peritumoral zone in which oedema and inflammation often occur (Ruiter et al. 2002). Although the role of CAFs in tumours has been well investigated, few studies have focussed on interactions that may occur between tumour cells and normal fibroblasts during the invasive stages. In a study looking at the peritumoral region of breast tumour tissue, however, a population of fibroblasts was observed that did not express α-SMA (Andarawewa et al. 2005). When in close proximity to tumour cells, these fibroblasts overexpressed MMP11 (stromelysin-3) which has been

associated with tumour invasion and poor patient prognosis (Basset et al. 1990; Chenard et al. 1996). Fibroblasts at the invasive front also appear to facilitate tumour cell invasion by forming tracks in the ECM for the tumour cells to move through, as demonstrated in squamous cell carcinomas (Gaggioli et al. 2007). This track formation occurs as a result of increased MMP activity and Rho/ROCK signalling to the fibroblast actomyosin cytoskeleton, and may explain how tumour cells that have not undergone EMT are still able to invade surrounding tissue and metastasize (Gaggioli 2008).

Although some studies have shown that fibroblasts are activated into myofibroblasts in the presence of tumour cells (Rønnov-Jessen et al. 1995; Kojima et al. 2010), not all fibroblasts are necessarily converted. In a study by Kojima et al. (2010), less than 50 % of normal fibroblasts mixed with MCF7-ras breast tumour cells and injected into mice were shown to express α-SMA, even after 242 days of co-culture. Shekhar et al. (2003) also showed that normal skin fibroblast spheroids co-cultured with breast tumour spheroids did not display increased levels of α-SMA, even though the tumour cells were seen to migrate into the fibroblast spheroids. Since the exact origins of CAFs are also still in question (Rønnov-Jessen & Bissell 2008; Hinz et al. 2007) more studies need to be undertaken to examine the specific role of normal fibroblasts in tumours and also to determine how invading tumour cells influence normal fibroblasts that they come into contact with at the invasive front.

1.3.1.3 Signalling between tumour cells and fibroblasts

Different regions of a tumour may have variations in ECM characteristics, metabolite availability and cellular distribution (Egeblad et al. 2010). Communication between tumour cells and stromal fibroblasts may therefore depend on the proximity of these cells to each other and may involve secreted factors and/or cell adhesion molecules (De Wever & Mareel 2003). Secreted factors include cytokines, chemokines and growth factors, which move into the surrounding ECM and can affect neighbouring cells over a short or long range (Fagotto & Gumbiner 1996). On the other hand, ECM and cell surface

receptors such as integrins and cadherins are involved in juxtacrine signalling between cells in close contact.

Previous literature suggests that there are a number of secreted factors involved in tumour-stroma cell signalling, including TGFβ, EGF (epidermal growth factor), HGF (hepatocyte growth factor) and stromal derived factor-1 (SDF-1/CXCL12). TGFβ stimulation can trigger increased expression of the chemokine, CXCL12, and its receptor, CXCL14, in fibroblasts and this plays a direct role in myofibroblast activation (Kojima et al. 2010). Furthermore, SDF-1 secreted by the activated fibroblasts has been shown to increase tumours growth rate and angiogenesis (Orimo et al. 2005; Kojima et al. 2010). On the other hand, Tyan et al. (2011) showed that levels of HGF were higher in CAFs than normal fibroblasts and that it was this secreted HGF that was responsible for stimulating tumour growth. Interestingly, factors secreted by MDA-MB-468 tumour cells were also able to stimulate HGF secretion from normal fibroblasts. MCF7-ras and MDA-MB-231 breast tumour xenografts were also shown to specifically recruit bone-marrow-derived human mesenchymal stem cells that were injected into the bloodstream of mice, and these cells are believed to also contribute to the myofibroblast population (Karnoub et al. 2007). The breast tumour cells also stimulated the mesenchymal stem cells to secrete CCN5 (RANTES) which, in turn, acted in a paracrine manner to enhance tumour motility, invasion and metastasis. Therefore, there are a number of complex interactions between tumour cells and fibroblasts/CAFs that contribute to tumour growth and invasion.

Although some secreted factors may convey their effect over large distances, others need close proximity with neighbouring cells to relay their signals successfully. Shephard et al. (2004) showed that keratinocytes were able to induce a myofibroblast phenotype with increased type I collagen gene expression in normal dermal fibroblasts, but that this only occurred after cells were directly co-cultured for four days. Close contact with fibroblasts stimulated keratinocytes to secrete IL-1 immediately after co-culture, which inhibited TGFβ-stimulated myofibroblast differentiation until day four. Direct co-culture of dermal fibroblasts with activated T-cells was also required for the down-regulation of type I and type III collagen gene expression in fibroblasts, since this effect did not occur when cells

were separated during co-culture (Rezzonico et al. 1998). Further analysis revealed that collagen levels in fibroblasts were negatively regulated by TNFα, IFNγ and IL1 produced by T-cells. The regulation of type I collagen gene expression in these studies could therefore be concentration dependent and/or there could be other cell surface receptors involved in mediating these effects.

Cell surface adhesion molecules such as integrins and cadherins are also involved in communicating signals from the ECM and other cells. An α and β sub-unit makes up the integrin dimer, with up to 24 α/β heterodimer combinations existing to allow specific interactions with ECM components (Ivaska & Heino 2011). Integrins act by binding to ECM components via their ectodomains while the smaller cytoplasmic domains bind to the cytoskeleton (Arnaout et al. 2007). This allows interactions and communication to take place between the cell and the surrounding matrix. Binding of integrins to ECM components can induce integrin clustering, resulting in a higher local concentrations of associated downstream signalling kinases and leading to the activation of these enzymes and downstream signalling components (Kim & Ginsberg 2011). Ligand-induced conformational changes in integrins can also results in the separation of the intracellular domains of the α and β sub-units, with resulting receptor activation (Kim & Ginsberg 2011; Du et al. 1991)

Integrins activate similar signalling pathways to those activated when growth factors bind to their receptors, and recent studies have shown that cross-signalling between integrins and growth factor receptors can occur (Eliceiri 2001; Bosman & Stamenkovic 2003; Comoglio et al. 2003). Adhesion molecules can act synergistically with growth factors to coordinate biological responses; for example, fibroblasts were unable to migrate in response to PDGF or EGF signals when the focal adhesion kinase (FAK) was not available (Sieg et al. 2000). FAK is usually involved in integrin-mediated signalling, but, in this case, efficient growth factor-mediated migration only occurred when FAK associated with both integrins and growth factor receptors. Adhesion molecules can also complex with growth factor receptors independently of growth factor binding to activate downstream receptor kinases; for example, the α2β1 integrin was shown to associate with and activate

the epidermal growth factor receptor (EGFR) on neighbouring epithelial-like adenocarcinoma cells at cell-cell contact sites, independently of EGF binding (Yu et al. 2000).

Integrins may also interact with other cell surface receptors, such as the immunoglobulin-type receptors ICAMs (intercellular adhesion molecules) and VCAMs (vascular cell adhesion molecules), as well as members of the ADAM (a disintegrin and metalloprotease) family found on adjacent cells (Bernstein 1998; Oberyszyn et al. 1998; Heino 2000; Danen 2005). Other adhesion molecules which are mainly involved in homotypic cell-cell interactions include the cadherin family of transmembrane glycoproteins (Alattia et al. 1999). E-cadherin is usually involved in maintaining close contact between neighbouring epithelial cells, but tumour cells often lose their ability to express E-cadherin while gaining N-cadherin expression. N-cadherin-mediated adhesion of melanoma cells to dermal fibroblasts and vascular endothelial cells has previously been shown to increase melanoma cell growth and facilitate tumour migration (Li et al. 2001).

A number of different signalling molecules and mechanisms may therefore be involved in tumour/fibroblast cross-talk, with close contact between these cells during tumour invasion allowing for interactions to take place that would not occur in normal tissue. These interactions may involve a number of different growth factors, cytokines, cell surface receptors and cell adhesion molecules and further research will need to be performed to elucidate the exact mechanisms involved and how these influence tumour invasion.

1.3.2 The role of the stroma in determining tumour prognosis

As previously discussed, gene expression studies performed on breast tumour tissue have facilitated further classification of these tumours into subtypes and therefore improve diagnostic capabilities. These gene expression studies have also been used to characterise the stromal component of breast tumours in an attempt to improve our understanding of the role that the stroma in breast tumour development and prognosis.

Since breast tumour development is often regarded as a wound-healing response gone wrong, Chang et al. (2004) investigated whether serum activation of fibroblast as observed during wound healing could be used to characterise tumours. Gene expression analysis identified a set of 512 genes, termed the fibroblast "core serum-response" (CSR), which were then used to classify a group of breast, lung, prostate, hepatocellular and gastric carcinomas tissue samples into a "wound activated" and "wound quiescent" subtype. Breast cancer patients with the activated CSR signature, which included most basal-like tumours, were found to have a poorer metastasis-free survival rate than those without the signature (Chang et al. 2005).

More recently, Finak et al. (2008) used stromal cells isolated from breast tumour tissue for gene expression analysis to create a "stroma derived prognostic predictor" (SDPP), with differences in immune responses, as well as angiogenic and hypoxic responses detected. When this profile was used to classify a set of breast tumour tissue samples, disease outcome could be predicted independently of traditionally used clinical prognostic methods as well as previously published expression-based predictors which used whole tumour tissue samples (including tumour cells and stromal cells) for creating these profiles. This predictor set also had increased prognostic power, which was further improved when this SDPP was combined with other existing predictors. In a separate study, Bergamaschi et al. (2008) classified primary breast tumours into four groups (ECM1-4) according to a set of differentially expressed ECM-related genes. When these predictors were used to classify an independent set of breast carcinoma samples, the ECM1 group proved to be useful for predicting specific biological and clinical behaviour. This group was originally characterised by the overexpression of genes for integrins and other cell-surface receptors, as well as metallopeptidases and some laminins. Basal-like tumours clustered more in the ECM1 group, while Luminal A and B tumours were found in all groups. However, patients with luminal type tumours classified as ECM1 were given a poorer prognosis, which may be a result of the high level of vascularization observed in ECM1 tumours. Usually patients diagnosed with luminal type breast cancer are placed in a 'good-prognosis' group, but the ECM prognostic set used in this study was able to further identify patients in this group with a poorer prognosis.

The results of these gene expression analyses as well as results of other studies looking at the relationship between tumour cells and the surrounding stroma provide valuable insights that will assist in improving the accuracy of breast cancer diagnosis as well as in identifying improved targeted therapeutic agents. Such therapeutic agents currently under development include antibodies or peptides that are able to interfere with interactions between the ECM and cell-surface integrins (Chung et al. 2005). Further analysis of the role of the tumour stroma in resistance to chemotherapeutic agents, hormones and radiation will also prove vital for the development of future therapeutics (Weigelt & Bissell 2008).

1.4 Type I Collagen - the major component of the ECM.

Fibroblasts produce most of the ECM components, including type I collagen. This fibrillar collagen is the main constituent of the ECM and plays an important part in giving strength and elasticity to connective tissue (Bhogal et al. 2005). Regulating the synthesis and degradation of this protein is therefore essential for maintaining tissue homeostasis and a number of positive and negative regulators of type I collagen gene expression have been identified.

In this section we discuss the literature on type I collagen, including the various factors involved in the regulation of type I collagen gene expression. Specific focus will be placed on the role of TGFβ and connective tissue growth factor (CTGF/CCN2) as positive regulators and that of interferons (IFNs) as a negative regulator of type I collagen gene expression, as well as the varying effects of MAPK signalling pathways on type I collagen regulation. Finally, we will look at how type I collagen is regulated in tumours and which factors influence this regulation.

1.4.1 The structure and function of type I collagen.

Collagens are the most abundantly found protein in vertebrates and this family consists of at least twenty eight members. These can be divided into the following categories: fibrillar and network-forming collagens, the FACITs (fibril-associated collagens with

interrupted triple helices), MACITs (membrane-associated collagens with interrupted triple helices), and MULTIPLEXINs (multiple triple-helix domains and interruptions) (Shoulders & Raines 2009).

Collagens are mainly responsible for maintaining tissue integrity and stability, as well as regulating cell polarity, migration and survival (van Kempen et al. 2005). They are expressed at high levels in areas of the body needing high tensile strength (e.g. skin, bone, tendons, heart, aorta), but at lower levels in most organs and non-structural tissues (Stefanovic 2005). They also play an important role in modulating cellular differentiation and morphogenesis during development and wound healing. Changes in collagen structure and metabolism are found in a number of inherited or acquired diseases such as cancer, fibrosis, osteogenesis imperfecta, osteoarthritis and atherosclerosis (Trojanowska et al. 1998; Varga & Jimenez, 1995).

As much as 90% of the collagen found in the body is type I collagen, which is synthesized mainly by fibroblasts, osteoblasts and odontoblasts (Verrecchia & Mauviel 2004; Ghosh 2002). This fibrillar collagen has a heterotrimeric structure and is composed of two α1(I) and one α2(I) polypeptide chains that are produced by the COL1A1 (17q21.31-22.05) and COL1A2 (7q21.3-22.1) genes, respectively (Retief et al. 1985). The α1(I) and α2(I) procollagen polypeptides are synthesized in a 2:1 ratio and undergo extensive post-translational modifications in the endoplasmic reticulum (ER) before being transported to the Golgi apparatus and secreted from the cell via secretory vesicles (Verrecchia & Mauviel 2004). Once in the extracellular matrix, the N- and C-propeptides are cleaved by specific proteases and cross-linking of the mature collagen molecule occurs, catalysed by lysyl oxidase, to form the collagen triple helix known as tropocollagen (Siegel et al. 1978; Shoulders & Raines 2009). The mature tropocollagen monomers then assemble into microfibrils and fibrils by covalent cross-linking formed between the telopeptides of one collagen molecule and the adjacent helical domains of another collagen molecule (Shoulders & Raines 2009) and it is this cross-linking that gives the collagen fibers their strength.

1.4.2 Transcriptional regulation of type I collagen gene expression.

The regulation of type I collagen synthesis occurs in both a tissue- and cell-specific manner and is tightly coordinated. It can occur at either a transcriptional, post-transcriptional, translational or post-translational level (Xu et al. 2006). Most previous studies have focused on the regulation of type I collagen production at a transcriptional level and have used transgenic mice generated with reporter genes driven by various segments of the mouse α1(I) or α2(I) procollagen promoters. Results have shown that different *cis*-acting elements modulate procollagen gene expression in different type I collagen-producing cell types and tissues. Studies in rats and humans produced similar results, suggesting conservation of *cis*-acting elements in different species (Rossert et al. 1995). There are, however, variations in transcriptional regulation of the mouse and human procollagen promoters (Ihn et al. 1996; Leaner et al. 2005). Because of the species and cell-type specific variations often observed in type I collagen regulation, this review will focus mainly on the regulation of type I collagen in normal human fibroblasts.

Transcriptional regulation of the COL1A1 and COL1A2 genes appear to be modulated by the binding of similar transcription factors, although the regulatory promoter regions have distinctly different structural arrangements (Boast et al. 1990). The exact controlling regions also appear to vary depending on cell type and tissue, and conflicting results have been obtained (Boast et al. 1990; Bornstein & Mckay 1988; Slack et al. 1991). Results from a number of transcriptional regulation studies indicate that maximal COL1A1 promoter expression in human fibroblasts only requires the -174 bp region of the proximal promoter, with a negative regulatory element found between -804 bp and -5.3 kb (Jimenez et al. 1994; Artlett et al. 1998; Boast et al. 1990; Bornstein & Mckay 1988; Hitraya & Jimenez 1996). Binding of three transcription factors (Sp1, Sp3 and CBF (CCAAT binding factor)) to four sites on the -174 to -84 bp promoter region is all that is required for basal COL1A1 gene expression (Chen et al. 1998) (Figure 2.1 A). CBF was shown to only bind to the inverted CCAAT box located at -100 to -96 bp, but not to the one at -125 to -121 bp (Saitta et al. 2000). Sp1 binds to both the -120 to -115 region (distal) and the -86 to -81 bp region (proximal), while Sp3 associates with a TCCTCC motif at -158 to -153 bp (Artlett et al. 1998; Jimenez & Saitta 1999; Saitta et al. 2000). Both CBF and Sp1 also

form protein-protein contacts with the TATA-associated factors (TAFs) and therefore interact with the initiation complex of the core promoter (Jimenez & Saitta 1999).

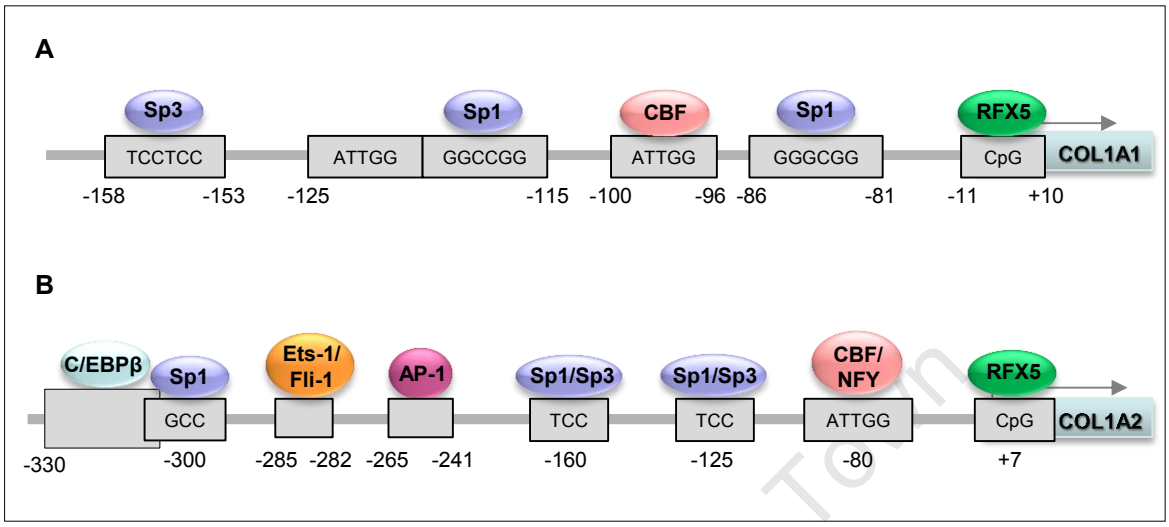

FIGURE 1.2 Transcription factors involved in regulating basal COL1A1 and COL1A2 promoter activity. (A) COL1A1 promoter activity is positively regulated by Sp1, Sp3 and CBF (CCAAT binding factor). Sp1 associates with the -120 to -115 bp as well as the -86 to -81 bp region of the promoter, while Sp3 associates with the -158 to -153 region. The inverted CCAAT box at -100 to -96 bp is bound by CBF. RFX suppresses COL1A1 promoter activity by associating with the -11 to +10 bp region (Adapted from Saitta et al., 2000). (B) Most of the transcription factors involved in regulatingCOL1A2 promoter activity bind to the -380 to +54 bp region of the promoter. Association of Sp1 with the GCC-rich region at -300bp and Sp1 or Sp3 with the TCC-rich region at -125 bp leads to increased COL1A2 promoter activity, while binding of Sp1 or Sp3 to the -160 bp TCC-rich region negatively regulates promoter activity. Binding of the CBF/NFY (CCAAT binding factor/nuclear factor Y) trimer to the inverted CCAAT box in the -80 bp region also positively regulates promoter activity. Ets-1 (positive regulator) and Fli-1 (negative regulator) compete for binding to the -285 to -282 bp region. C/EBPβ (CCAAT/Enhancer binding protein β) also acts as a positive regulator of basal COL1A2 promoter activity, while AP-1 and RFX5 are negative regulators..

Sp1 family members play an important role in both basal and TGFβ induced expression of the COL1A1 and COL1A2 genes (Ihn et al. 1996; Artlett et al. 1998; Pogulis & Freytag 1993; Verrecchia et al. 2001b; Tamaki et al. 1995). The -380 to +54 region of the COL1A2 promoter, which constitutes the minimal proximal promoter, contains three sequences which are essential for Sp1 binding and basal COL1A2 promoter activity (Boast et al. 1990; Inagaki et al. 1994) (figure 1.2 B). The site at -300 bp is bound only by Sp1, while the two TCC-rich boxes at -160 (distal) and -125 bp (proximal) can associate with Sp1 and Sp3. The -300 bp Sp1 site and the proximal TCC-rich box are involved in positive regulation of COL1A2 promoter activity while binding to the distal TCC-rich box results in negative regulation of both the downstream TCC-rich box and the upstream GCC-rich elements (-300bp region). However, this association does not affect the activity

of the inverted CCAAT motif at -80 bp, which associates with a CBF/NFY (CAAT binding factor/nuclear factor Y) trimer (Ihn et al. 1996; Ramirez et al. 2006). Other functionally distinct DNA elements in the -300 bp region bind AP1, Ets1, and C/EBPβ (CCAAT/enhancer binding protein beta) proteins (Ramirez et al. 2006). The Ets1 transcription factor binds to the -285 to -282 bp region of the COL1A2 promoter to positively regulate transcription (Czuwara-Ladykowska et al. 2002). However, the negative regulator Fli-1 competes with Ets-1 for binding to both the COL1A1 and COL1A2 promoters in normal dermal fibroblasts to regulate the expression of both genes (Czuwara-Ladykowska et al. 2002; Kubo et al. 2003). C/EBP-β interacts with the -330 to -303 bp region of the COL1A2 promoter, although the role of this protein in regulating COL1A2 transcription depends on the conditions under which it is expressed. Overexpression of C/EBPβ in foreskin fibroblasts was shown to induce COL1A2 promoter expression (Ghosh 2002); on the other hand, C/EBPβ is a negative regulator of the TGFβ response and a positive regulator of TNFα repression of COL1A2 gene expression (Greenwel et al. 2000). Binding of AP-1 to a site between -265 and -241 bp also appears to play a role in COL1A2 promoter regulation (Chung et al. 1996), and association of RFX5 with the COL1A2 (+ 7 bp) and COL1A1 (-11/+10 bp) promoter negatively regulates the transcription of both genes (Sengupta et al. 2002; Sengupta et al. 2005).

1.4.3 Cytokines, growth factors and signalling pathways involved in type I collagen gene regulation

Extracellular signalling molecules such as cytokines and growth factors play an important role in regulating type I collagen production via a number of signal transduction pathways (Verrecchia & Mauviel 2004). TGFβ is a well-studied cytokine involved in type I collagen up-regulation (Dijke & Hill 2004; Verrecchia & Mauviel 2004; Massague & Wotton 2000), but other factors such as insulin-like growth factor (IGF1), endothelin 1 (ET1), IL4, and IL13 also stimulate type I collagen production (Ramirez et al. 2006; Serpier et al. 1998; Buttner et al. 2004). Well-studied negative regulators of type I collagen gene expression include TNFα (Inagaki et al. 1995; Kouba et al. 1999; Mori et al. 1996), IFNγ (Higashi et al. 2003a; Xu et al. 2004) , IL1 (Mauviel et al. 1991) and IL10 (Reitamo et al.

1994), and these all function by antagonizing TGFβ signalling (Verrecchia & Mauviel 2002; Higashi et al. 1998).

1.4.3.1 TGFβ-mediated regulation of type I collagen gene expression.

TGFβ regulates a number of different cellular processes in both embryonic development and in the adult organism. These include cell survival, differentiation, migration, adhesion, and synthesis of ECM components (Inagaki & Okazaki 2007). This growth factor is the most potent stimulator of type I collagen gene expression and plays an important role in ECM synthesis and remodeling. Overexpression of TGFβ has also been associated with a number of fibrotic diseases (Leask & Abraham 2004).

TGFβ is secreted as a latent protein complex and associates with ECM components such as type IV collagen, fibronectin and fibrillin. TGFβ is activated during tissue remodeling by factors such as MMPs, αvβ6 integrin and thrombospondins. It then binds to the TGFβ type II receptor (TGFBRII) on the cell surface, resulting in the activation of the Smad signalling pathway. TGFBRII phosphorylates TGFBRI (TGFβ type I receptor) which, in turn, phosphorylates two serine residues in the C-terminal motif of Smad2 and Smad3. These are known as the receptor-activated Smads (R-Smads) and form hetero-oligomers with Smad4 (Co-Smad) before translocating to the nucleus. Here the Smad complex regulates the transcription of a number of genes either by directly binding to the CAGAC consensus sequence or through interaction with transcription coactivators such as p300 and CBP (Ramirez et al. 2006).

The -330 to -255 region of the COL1A2 promoter is known as the TGFβ-responsive element (TβRE) consisting of Box 3A and Box B (figure 1.3). In response to TGFβ, Box 3A-bound Sp1 interacts with Box B-bound Smad3 and p300/CBP coactivators, leading to stimulation of COL1A2 gene transcription (Inagaki & Okazaki 2007; Zhang et al. 2000). Box 5A, upstream and overlapping Box 3A, binds C/EBPβ, which suppresses TGFβ-stimulated COL1A2 promoter activity (Inagaki & Okazaki 2007). The COL1A1 promoter has a TGFβ-responsive element in the -174 to -84bp region which is known to contain Sp1

binding sites (Gaidarova et al. 2002; Jimenez et al. 1994)). The Smad signalling pathway also appears to be involved in mediating the COL1A1 promoter response to TGFβ response, since overexpression of Smad3 increases COL1A1 promoter activity (Verrecchia et al. 2001a). However, further studies need to be performed to determine how transcription factors associate with the COL1A1 promoter during TGFβ stimulation.

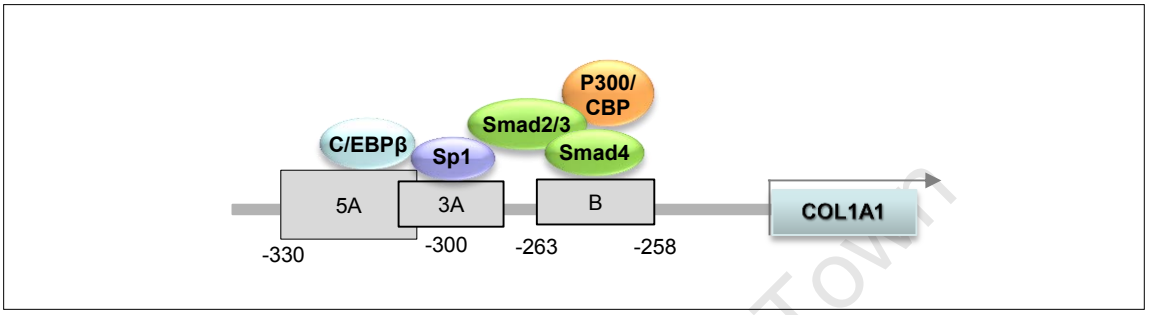

FIGURE 1.3 The TGFβ-responsive element (TGFβ-RE) is involved in COL1A2 promoter activation by TGFβ. TGFβ stimulation results in the activation of Smad2 and Smad3, which form a complex with Smad4 before translocating into the nucleus. The Smad complex then associates with p300/CBP and binds to the COL1A2 promoter in the -263 to -258 bp region (box B). Further association of the Smad complex with Sp1, bound to the -300 bp region (box 3A), is required for complete COL1A2 promoter activity. C/EBPβ negatively regulates TGFβ stimulated COL1A2 promoter activity by associating with the -330 to -303 bp region (box 5A). (Adapted from Inagaki & Okazaki, 2007).

Binding of TGFβ to its receptor complex also stimulates production of Smad7, which then acts as a negative regulator of the TGFβ response by either interfering with Smad-receptor or Smad-Smad interactions (Itoh & ten Dijke 2007; Massague et al. 2005). Smad7 can compete with R-Smads for binding to TGFβRI and thereby prevent their phosphorylation (Hayashi et al. 1997; Nakao et al. 1997). Shi et al. (2004) reported that Smad7 can also induce dephosphorylation and deactivation of TGFβRI itself by stabilizing and recruiting the protein phosphatase PP1/GADD34 complex to the receptors. Smad7 can also recruit the ubiquitin ligase Smurf1 to the activated TGFβ receptors, leading to eventual degradation of the receptor (Heldin et al. 2009; Ramirez et al. 2006). In human skin fibroblasts, overexpression of Smad7 may lead to a decrease in basal COL1A2 promoter activity as well as TGFβ-stimulated COL1A1 and COL1A2 promoter activity (Chen et al. 1999).

TGFβ is believed to be one of the important players in the tumour environment. In normal and premalignant tissue TGFβ is responsible for inhibiting epithelial cell growth (differentiation, apoptosis, cytostasis) and also influences the stroma by suppressing inflammation and stroma-derived mitogens (Heldin et al. 2009; Massague 2008). It is also involved in fibroblasts activation to myofibroblasts during conditions such as wound healing (Shephard et al. 2004). However, TGFβ's role in cancer is still controversial. Initially it is believed to act as a tumour suppressor, inhibiting cell growth and inducing apoptosis. However, at later stages TGFβ secretion by tumour cells may trigger surrounding cells to secrete cytokines that result in tumour cell resistance to growth arrest stimuli and EMT induction (Bierie & Moses 2006). TGFβ then acts as a tumour promoter that may be used by the tumour cells to initiate immune evasion, promoting angiogenesis and facilitate tumour invasion and metastasis (Heldin et al. 2009; Massague 2008).

The importance of TGFβ signalling in tumour-fibroblast interactions was highlighted in a recent mouse study (Bhowmick et al. 2004b), involving transplantation of fibroblasts with a targeted deletion of TGFBRII into mice together with breast tumour cells. The resulting tumours were found to be more aggressive, with an increased number of metastases compared to those transplanted with normal fibroblasts. Increased production of TGFα and hepatocyte growth factor (HGF) by these TGFβ-unresponsive fibroblasts was shown to play a role in this accelerated tumour cell growth observed. These results suggest that TGFβ would usually act by limiting the production of these mitogenic factors by fibroblasts, and that unresponsiveness of fibroblasts to TGFβ signalling facilitates tumour development.

1.4.3.1.1 CCN2 as a mediator of TGFβ's effect on type I collagen gene expression.

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN family and these extracellular matrix-associated (matricellular) proteins are involved in mediating cellular responses to environmental factors and stimuli by their interactions with cell surface receptors (Chen & Lau 2009). CCN2 therefore acts mainly as a modulator and co-

mediator of the biological effects induced by other molecules rather than stimulating responses directly (Blom et al. 2002); for example, CCN2 has been shown to enhance TGFβ-stimulated responses which includes increasing type I collagen gene expression (Leask et al. 2003; Quan et al. 2009; Shi-wen et al. 2006). Although originally called CTGF, this protein is in fact not a growth factor and will hereafter be referred to as CCN2.

The development of a CCN2-null mouse model has revealed the essential role of CCN2 in regulating the production of cartilage ECM during development (Ivkovic 2003). CCN2 knockout mice die soon after birth, mainly as a result of respiratory failure caused by skeletal defects, and fibroblasts from these mice had a reduced ability to migrate, form actin stress fibres, as well as attach to and contract the surrounding matrix (Blom et al. 2002; Shi-wen et al. 2008). These fibroblasts also express lower levels of certain pro-angiogenic cytokines and have a decreased ability to produce type I collagen and α-SMA when stimulated with TGFβ. CCN2 is constitutively expressed at low levels in adult tissues and biological fluids (Oliver et al. 2010), but expression is increased during inflammation and wound healing as well as in most fibrotic disorders (Khoo et al. 2006; Leask et al. 2009; Shi-wen et al. 2000). In fact, constitutive CCN2 expression is a marker of pathological fibrosis (Chen et al. 2001; Holmes et al. 2001) and most CCN2 studies have focussed on their role in fibrotic disorders with less information available on their function in normal adult tissue (Mason 2009; Oliver et al. 2010).

CCN family member genes are highly conserved among species. The CCN2 gene itself has five exons which are predicted to code for a signal peptide and four domains (see figure 1.4) (Blom et al. 2002). The first exon is translated into a 37 amino acid secretory signal peptide that is involved in the export of CCN2 from the ER to the Golgi. Exon II encodes for the first domain which is very similar to insulin-like growth factor binding protein (IGFBP) and is able to bind IGF with low affinity, while the second domain, encoded by exon 3, is very similar to the Von Willebrand factor type C repeat and is thought to play a role in oligomerisation. The third domain is a thrombospondin type I domain (TSP1) which contains a cell attachment motif, and exon 5 encodes the C-terminal domain which has a cystine knot motif also found in other growth factors (e.g. TGFβ) and

which is involved in cell surface receptor binding. Since all four domains are highly similar to other known proteins, it has been suggested that the CCN2 gene evolved as a result of duplication and shuffling of exons originating from older genes (Blom et al. 2002).

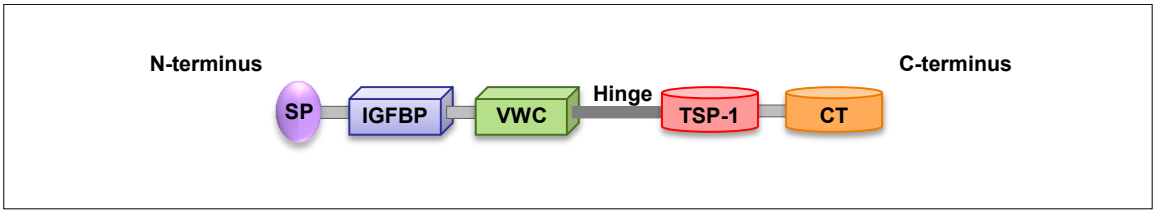

FIGURE 1.4 CCN2 protein domain structure. The CCN2 protein consists of an N-terminal secretory signal peptide (SP) and 4 domains. IGFBP, Insulin growth factor binding protein; VWC, Von Willebrand factor type C repeat; TSP-1, Thrombospondin type 1 domain; CT, C-terminal domain.

CCN2 can either be secreted through the Golgi apparatus into the stroma or remain attached to the cell surface and ECM (Blom et al. 2002; Chen et al. 2001). CCN2 is expressed as a 38-kDa protein, but cleaved forms of the CCN2 protein have also been observed in different cell types, tissues and body fluids (Wahab et al. 2001). The flexible hinge region joining the N-terminal domain (containing the IGFBP and VWC motifs) to the C-terminal domain (containing the TSP-1 and CT motifs) can undergo protease digestion resulting in products of 16-20 kDa that still maintain their biological activity and are capable of acting independently of each other (Grotendorst & Duncan 2005). The N-terminal domain, for example, interacts with IGF-2 to mediate differentiation and collagen synthesis while the C-terminal domain facilitates cell proliferation in partnership with EGF (Grotendorst & Duncan 2005). CCN2 can also be cleaved into monomers (10-12 kDa), consisting of only one motif, and homodimers (~70-80 kDa) as well as phosphorylated forms of CCN2 (~44 kDa) have also been observed (Ball et al. 2003; Khoo et al. 2006; Holbourn et al. 2008; Tikellis et al. 2004; Wahab et al. 2001). Much of the variation in CCN2 forms appears to be cell type specific (Blom et al. 2002).

Although a unique CCN2 receptor has not been found, this protein appears to mediate much of its effects through cell adhesion receptors such as integrins and heparin sulphate proteoglycans (HSPG) (Chen & Lau 2009; Leask & Abraham 2004). Association of

CCN2 with these cell surface proteins leads to the activation of intracellular signalling molecules such as FAK (focal adhesion kinase), MAPK p42/44, PI3K and Rac which can in turn promote cell survival, proliferation, adhesion, and extracellular matrix production (Grotendorst et al. 2004; Mason 2009). CCN2 may also form complexes with other growth factors, such as IGF, and binds either to the corresponding growth factor receptor or to an as yet unknown receptor (Grotendorst et al. 2004). Secreted CCN2 may also be internalized again and undergo endosomal degradation, mediated by binding of the C-terminal CT motif of CCN2 to the low density lipoprotein receptor related protein (LRP)/α2-macroglobulin receptor (α2-MR) (Chen et al. 2001; Segarini et al. 2001).

Basal expression of CCN2 varies according to cell type, but it is constitutively expressed at low levels in normal human skin *in vivo* and in primary human skin fibroblasts (Quan et al. 2009). CCN2 expression is regulated mainly at the transcriptional level (Grotendorst et al. 1996; Leask et al. 2003) and one of the most potent inducers of CCN2 gene expression in fibroblasts, but not in epithelial cells, is TGFβ (Hishikawa et al. 1999; Leask et al. 2003). CCN2 also acts as a co-mediator of TGF-β's ability to promote type I collagen synthesis, with *ccn2-/-* embryonic fibroblasts being unable to induce type I collagen synthesis in response to TGFβ (Shi-wen et al. 2006). An important relationship therefore exists between TGFβ, CCN2 and type I collagen, and in aged human skin (≥ 80 years) the expression of all three of these proteins is co-ordinately reduced when compared to levels in younger skin samples (21-30 years) (Quan et al. 2009).

Regulation of CCN2 gene expression by TGFβ involves the association of a Smad3/Smad4 complex with a Smad binding element (SBE) on the CCN2 promoter, with no involvement of either Smad2 or p300 (Holmes et al. 2001). The CCN2 promoter also has a TGFβ response element (TGFβRE) which appears to be important for the regulation of basal CCN2 gene expression in fibroblasts, and is therefore also called the basal control element (BCE-1) (Holmes et al. 2001). Other signalling pathways that are involved in basal and TGFβ-mediated CCN2 up-regulation include the ras/MEK/ERK and protein kinase C (PKC) pathways (Blom et al. 2002; Leask et al. 2003). CCN2 gene expression is also positively regulated by endothelin-1, angiotensin II, glucose and thrombin, while TNFα,

IL-1, prostaglandin E2, lefty and cAMP (cyclic adenosine monophosphate) act as negative regulators (Lin et al. 1998; Khoo et al. 2006; Mason et al. 2002; Nowinski et al. 2002).

Although CCN2 is known to enhance TGFβ-mediated up-regulation of type I collagen gene expression, the exact mechanism involved in this regulation is not well understood (Quan et al. 2009; Shi-wen et al. 2006). However, changes in CCN2 gene expression have previously been linked to changes in type I collagen gene expression (Ponticos et al. 2009; Shi-wen et al. 2006; Uchio et al. 2004), with overexpression of CCN2 in dermal fibroblasts resulting in increased COL1A2 gene expression, with specific involvement of the -376/+54 region of the promoter (Shi-wen et al. 2000). In lung epithelial cells, CCN2 increased TGFβ binding to its receptor complex, with a resulting increase in Smad2 phosphorylation and Smad-dependent gene transcription (Abreu et al. 2002). However, Smad-dependent signalling was not found to be directly involved in CCN2-mediated regulation of type I collagen in normal skin fibroblasts (Quan et al. 2009). Here, knockdown of CCN2 caused a significant down-regulation in COL1A2 promoter activity, but neither abrogation nor overexpression of CCN2 affected Smad2 or Smad3 phosphorylation.

CCN2 is thought to play a role in cancer cell migration, invasion and angiogenesis (Chu et al. 2008; Shi-wen et al. 2008). However, the exact role of this protein in cancer is still not well understood, and both positive and negative correlations have been drawn between CCN2 levels and tumour progression (Chu et al. 2008; Cicha & Goppelt-Struebe 2009). This may indicate that the action of CCN2 in cancer is context-dependent (Oliver et al. 2010). Increased expression of CCN2 has been detected in a number of cancers, including esophageal, melanoma and pancreatic cancer (Koliopanos et al. 2002; Kubo et al. 1998; Wenger et al. 1999). One study on breast tumour samples found that ~55% of samples had higher than normal CCN2 mRNA levels (Xie et al. 2001). However, in a separate study, significantly lower than normal CCN2 mRNA and protein expression was observed in breast tumour samples and this was correlated with poor prognosis, increased risk of metastasis and higher mortality rate (Jiang et al. 2004). CCN2 also formed part of a "bone metastasis gene signature" that cooperatively facilitated metastasis of MDA-MB-231 cells to bone in a mouse model (Kang et al. 2003). However, it is important to

distinguish between CCN2 expression in tumour cells themselves versus expression in surrounding stromal cells such as fibroblasts. Since regulation of this gene is cell-type specific and CCN2's associations with ECM proteins may vary, the function of CCN2 could also differ according to cell type.

1.4.3.2 Interferon-mediated regulation of type I collagen gene expression.

Interferons (IFNs) are proinflammatory cytokines that play an important role in innate and acquired immune responses (Leask & Abraham 2004). The interferon family consists of type I, II and III IFNs. Type I IFNs include IFNα and IFNβ, and are usually produced as a first line of defense against infections, where they function by limiting the spread of pathogens in the first few days of infection and by stimulating the adaptive immune response (Zhou et al. 2007). IFNγ, the only type II IFN, is usually produced by T-cells and NK cells as part of the adaptive immune response, while the recently discovered type III IFNs (IFNλ1, IFNλ2 and IFNλ3) function similarly to type I IFNs but act through a different cell surface receptor (Ghosh et al. 2001; Zhou et al. 2007). Both type I and type II IFNs have been shown to down-regulate type I collagen gene expression via the JAK/Stat signalling pathway, but more studies have focused on IFNγ's role as an antifibrotic cytokine (Buttner et al. 2004; Duncan & Berman 1985; Inagaki et al. 2003; Sengupta et al. 2002; Sengupta et al. 2005).

Although both type I and type II interferons activate the JAK/Stat signalling pathway, they generally use different mechanisms (figure 1.5). Association of type I IFNs with the IFN-α/β receptor (IFNαR1 & IFNαR2) causes JAK1 and TYK2 to bind to the receptor complex and induce tyrosine phosphorylation of Stat1α/β and Stat2. The Stat1/2 dimer associates with IRF-9 (IFN regulatory factor nine) to form the ISGF3 complex which is translocated into the nucleus (Ihle & Kerr 1995; Schindler et al. 2007). Here, the ISGF3 complex binds to interferon-stimulated response elements (ISREs) ($AGTTTN_3TTTCC$) on specific gene promoters resulting in the transcription of the relevant genes. When IFNγ binds to its receptor (IFNγR1 and IFNγR2), JAK1 and JAK2 are activated and, in turn, induce tyrosine phosphorylation of Stat1α. These activated Stat1 proteins form dimers that translocate to

the nucleus where they activate genes containing a γ-activation sequence (GAS) (TTTCCNGGAAA) in their promoters. Type I IFN induction can also cause Stat1 homodimer formation, but usually to a lesser extent than that found in the ISGF3 complex (Sadzak et al. 2008).

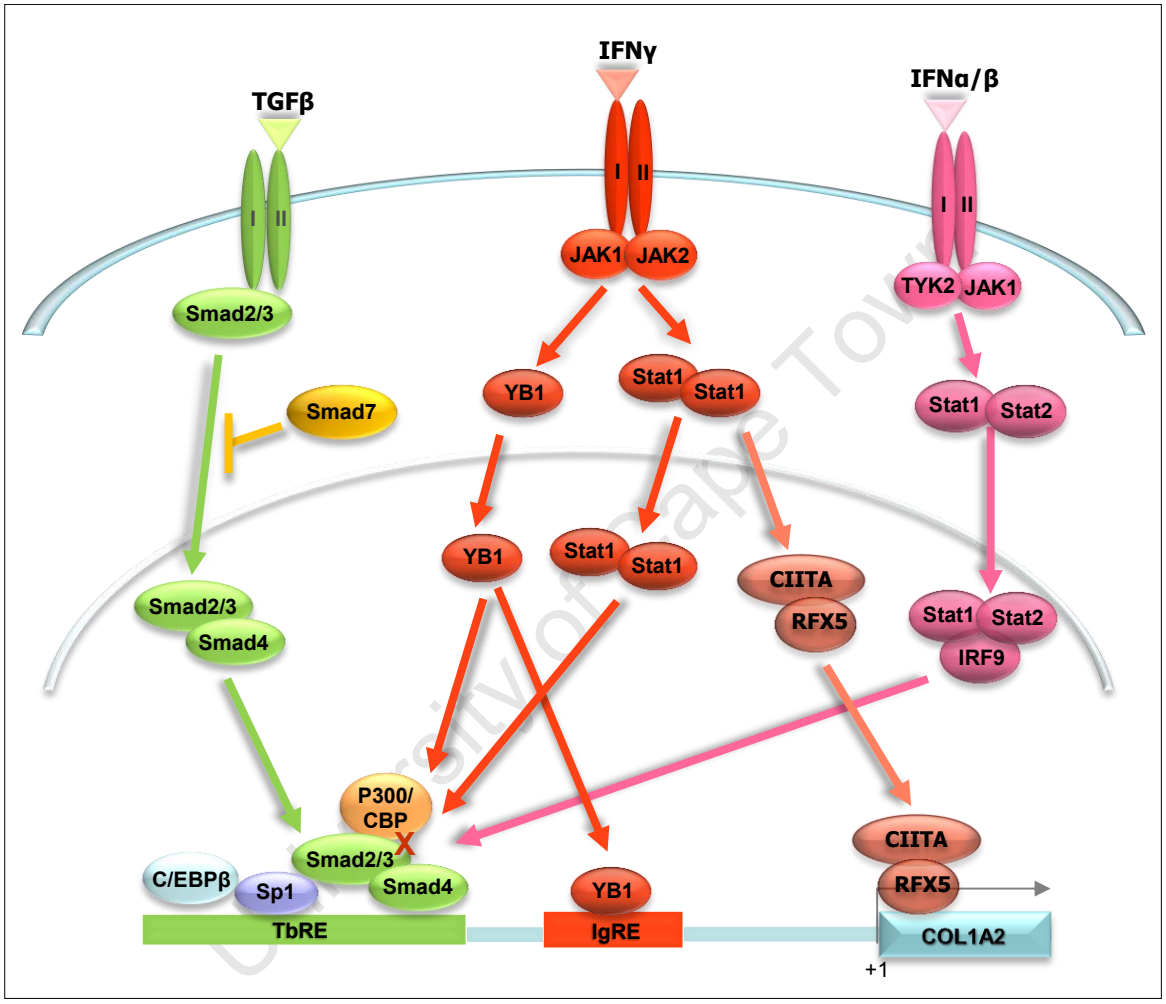

FIGURE 1.5 The antagonistic effects of interferons (IFNs) on TGFβ-stimulated COL1A2 transcription. Binding of TGFβ to its receptors stimulates Smad2 and Smad3 phosphorylation, and these Smads form a complex with Smad4 before translocating to the nucleus. Here, the complex interacts with p300 and stimulates COL1A2 transcription by binding to the TGFβ response element (TbRE) of the promoter. Binding of IFNγ to its receptors activates the JAK/Stat signalling pathway, resulting in Stat1 phosphorylation and dimerization and/or activation of the Y Box binding protein (YB-1). Both these factors antagonize TGFβ-stimulated COL1A2 promoter activity by preventing interaction of Smad3 with p300. YB-1 also negatively regulates COL1A2 promoter activity by binding to the interferon gamma response element (IgRE) on the COL1A2 promoter. Binding of the RFX5/CIITA complex to the +7 region of the COL1A2 promoter also inhibits transcription. IFNα and IFNβ can also negatively regulate COL1A2 promoter activity by activating JAK/Stat signalling. IFNα-dependent regulation involves activated Stat1, which prevents p300/Smad3 association with the COL1A2 promoter.

COL1A2 promoter regulation by IFNγ has been well studied, and has mostly focussed on the role of Stat1 in antagonizing TGFβ-mediated stimulation of type I collagen gene expression. IFNγ-stimulation of fibroblasts does not affect Smad3 or Smad4 expression or localisation (Gosh et al. 2001), however activated Stat1 is able to compete with Smad3 for binding to p300/CBP, which is essential for COL1A2 promoter activation by TGFβ (Ghosh et al. 2001). IFNγ has also been shown to up-regulate Smad7 gene expression during skin wound healing (Ishida et al. 2004) and also in epithelial and hepatic stellate cells (Ulloa et al. 1999; Weng et al. 2007), although Ghosh et al. (2001) did not observe any changes in Smad7 gene expression in fibroblasts stimulated with IFNγ. These discrepancies in results may be a result of variations in cell lineage-specific interactions. IFNγ-mediated repression of COL1A1 gene expression in skin fibroblasts appears to involve the -129 to -107bp region of the COL1A1 promoter as well as the transcription factor IRF-1 (interferon regulated factor 1), although the exact mechanism of action is not known (Yuan et al. 1999).

IFNγ may also inhibit type I collagen gene expression in a Stat1-independent manner. The transcription factor YB-1 is also activated by IFNγ and negatively regulates COL1A2 promoter activity by preventing p300/Smad3 interactions, or by binding directly to the interferon gamma response element (IgRE) on the COL1A2 promoter (Higashi et al. 2003a; Higashi et al. 2003b). IFNγ stimulation has also been shown to increase binding of RFX5, in association with CIITA, to the transcription start site (+7) of the COL1A2 promoter (Fang et al. 2009; Sengupta et al. 2002; Sengupta et al. 2005; Xu et al. 2003). COL1A1 promoter activity can also be negatively regulated by binding of the RFX5/CIITA complex to the -11/+10 promoter region in response to IFNγ (Sengupta et al. 2005).

Although IFNγ down-regulates both COL1A1 and COL1A2 gene expression, much less is known about the role of type I IFNs in regulating type I collagen gene expression. IFNα has been shown to negatively regulate COL1A2 promoter activity by associating with p300 and preventing its interaction with Smad3 (Inagaki et al. 2003). IFNβ can also

negatively regulate type I collagen synthesis (Duncan & Berman 1985), however further studies need to be performed to establish the mechanisms involved.

1.4.3.3 Regulation of type I collagen by MAPK signalling pathways.

MAP kinases are a group of serine-threonine specific protein kinases involved in conveying various extracellular signals from the cell surface to the nucleus. MAPK signalling pathways play an essential role in controlling a number of cellular responses, including cell proliferation, differentiation and death (Bardwell 2006). The three major MAPK subfamilies are the extracellular signal-regulated kinases (ERK1 and ERK2), the stress-activated protein (SAP) kinases (c-Jun N-terminal kinases – JNK1, JNK2 and JNK3) and the p38 MAP kinases (α, β, δ, γ) (Javelaud & Mauviel 2005) (figure 1.6). MAPKs are phosphorylated by MAPK kinases (MAPKKs) which, in turn, are activated by MAPK kinase kinases (MAPKKKs). Membrane-associated kinases, such as growth factor or cytokine receptors, activate these signalling pathways and downstream targets include a number of different nuclear kinases and transcription factors. Both positive and negative regulation of type I collagen by MAPKs have previously been observed, and some studies have shown conflicting results which suggest that regulation occurs in a cell-type specific manner (Leask & Abraham 2004; Javelaud & Mauviel 2005).

1.4.3.3.1 The MEK/ERK signalling pathway

The MEK/ERK signalling pathway can be activated in response to growth factors, mitogens and G-coupled protein receptors (GPCRs). ERK1 and ERK2 are phosphorylated by MEK1 and MEK2 (MAPKK's) which are, in turn, activated by Raf-1 (a MAPKKK). The membrane-bound onco-protein Ras is one of the proteins that can activate Raf-1 after being induced by the binding of EGF to its receptors (Javelaud & Mauviel 2005).

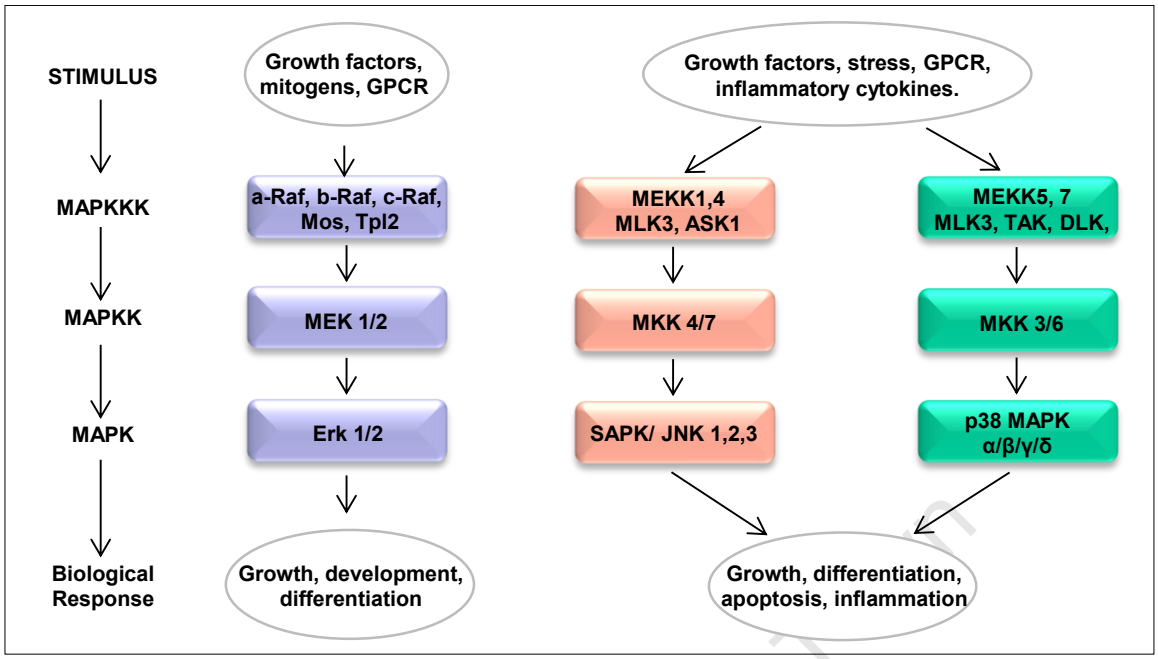

FIGURE 1.6 MAP kinase signalling pathways potentially involved in tumour-mediated type I collagen down-regulation. The MAPK pathways include the MEK/ERK, SAPK/JNK and p38 MAPK signalling pathways. These signalling pathways convey extracellular signals to the nucleus by means of a signalling cascade involving activation of a MAPK by a MAPKK which is, in turn, is activated by a MAPKKK in response to cell surface receptor signals. (Adapted from www.cellsignal.com).

The MEK1-ERK1/2 signalling pathway plays an important role the regulation of type I collagen steady-state mRNA levels in dermal fibroblasts. Phosphorylated ERK1/2 has been observed in quiescent dermal fibroblasts and inhibition of MEK/ERK activation results in a modest increase in both COL1A1 and COL1A2 mRNA levels with increased stabilization of COL1A1 mRNA levels (Sato et al. 2004). Addition of EGF (Mimura et al. 2006), C2-ceramide (Reunanen et al. 2000) and lysophosphatidic acid (LPA) (Sato et al. 2004) to dermal fibroblasts also negatively regulates type I collagen gene expression in a MEK/ERK-dependent manner. EGF stimulation resulted in decreased type I collagen mRNA stability with no effect on COL1A2 promoter activity, while synthetic C2-ceramide inhibited COL1A2 promoter activity as well as type I collagen mRNA levels and stability. Addition of the extracellular phospholipid LPA to fibroblast inhibited TGFβ-mediated up-regulation of type I collagen gene expression in an ERK-dependent manner, and also decreased type I collagen mRNA stability.

These results suggest that the MEK/ERK signalling pathway is involved in negative regulation of type I collagen gene expression. However, Li et al. (2009) recently showed

that ERK2, and not ERK1, is involved in increasing type I collagen synthesis after TGFβ stimulation of NIH/3T3 fibroblasts. Addition of IL-4 or IL-13 to dermal fibroblasts also increases type I collagen promoter activity in an ERK-dependent manner (Bhogal & Bona 2008). The effect of MEK/ERK signalling on type I collagen gene expression may therefore be dependent on interactions with other signalling pathways and/or may involve differential activation of either ERK1 or ERK2.

1.4.3.3.2 The JNK signalling pathway

JNK signalling is often activated in response to extracellular stress signals and plays a role in both the innate and adaptive immune response (Huang et al. 2009). The regulation of this pathway is very complex and may be influenced by a number of upstream kinases such as TGFβ-activated kinase 1 (TAK1), MEK1 and MLK3 (Johnson & Lapadat 2002; Huang et al. 2009). Activated JNK is translocated to the nucleus where it phosphorylates a number of transcription factors, including JunB, p53, and Stat3. JNK is also the only MAPK known to phosphorylate c-Jun, which forms the main component of the AP-1 complex (Javelaud & Mauviel 2005).

The role of the JNK signalling pathway in type I collagen regulation appears to be context dependent. TGFβ stimulation can up-regulate the expression of c-Jun and JunB in a Smad-dependent manner and both c-Jun and JunB then form part of a negative autoregulatory feedback loop by binding to Smad3 and thereby preventing the association of Smad3 with the Smad binding elements (SBE) in TGFβ-responsive promoters such as the COL1A2 promoter (Verrecchia & Mauviel 2004; Verrecchia et al. 2001a). However, TGFβ activation of the JNK signalling pathway is also involved in the induction of a myofibroblast phenotype with a resulting increase in type I collagen gene expression, as demonstrated in studies performed on embryonic mice fibroblasts (Liu et al. 2007). Myofibroblast activation involved upstream focal adhesion kinase (FAK) and increased ECM contraction, which suggests that activation of the FAK/JNK pathway by TGFβ could induce a matrix-remodelling phenotype with cross-talk between TGFβ signalling and adhesive signalling cascades occurring.

To complicate matters even further, the pro-inflammatory cytokines TNFα and IL-1β also activate the JNK signalling pathway in order to antagonize TGFβ-induced type I collagen gene expression via a mechanism involving the sequestration of p300 by cJun or JunB (Dennler et al. 2000; Verrecchia et al. 2000; Verrecchia et al. 2003). Decreased levels of p300 are therefore available for association with the Smad complex on the COL1A2 promoter, resulting in decreased COL1A2 gene transcription. The regulation of type I collagen gene expression by the JNK signalling pathway is therefore quite complex and appears to vary depending on signals obtained from a number of different cell surface receptors.

1.4.3.3.3 The p38 MAPK signalling pathway

The p38 MAP kinase pathway is activated in response to physical stress signals (e.g. osmotic shock, heat, and UV light) and appears to play an important role in regulating inflammation. The proinflammatory cytokines such as TNFα and IL1 can trigger p38 MAPK signalling activation, however anti-inflammatory TGFβ can also activate this pathway (Huang et al. 2009; Katsoulidis et al. 2005; Zhang et al. 2007a). Once activated, p38 can phosphorylate a number of downstream kinases and transcriptions factors such as ATF-2 (activating transcription factor 2), Stat1 and COX2 (cyclooxygenase 2) (van Boxel-Dezaire et al. 2006; Zhang et al. 2007b).

A number of studies have shown that type I collagen gene expression is up-regulated in response to p38 MAPK activation (Inagaki & Okazaki 2007). In dermal fibroblasts stimulated with TGFβ, activation of p38 MAPK signalling enhanced the association of Smad3 with p300/CBP coactivators and therefore increased the expression of type I collagen (Abecassis et al. 2004; Sato et al. 2002). Upstream TAK1 appeared to be involved in activating p38 in response to TGFβ, since blocking TAK1 expression prevented TGFβ-stimulated type I collagen up-regulation (Hanafusa et al. 1999; Ono et al. 2003; Yamaguchi et al. 1995). Nuclear p38 can also phosphorylate ATF-2 which forms a

complex with Smad4 in response to TGF-β stimulus (Hanafusa et al. 1999). However, the role of this interaction in type I collagen regulation is not yet known.

Although p38 up-regulates type I collagen gene expression in response to TGFβ stimulation, the role of p38 in IFN-mediated regulation of type I collagen has not previously been examined. The p38 signalling pathway is activated in response to both IFNα and IFNγ via a mechanism involving Stat1 up-regulation and serine phosphorylation (Goh et al. 1999; Uddin et al. 1999). However, further work will need to be performed to determine whether IFN-stimulated p38 activation has any effect on type I collagen regulation.

1.4.4 Type I collagen gene regulation in tumour development.

Studies that have analyzed type I collagen levels in tumour tissue have shown variations in results. In many types of solid tumours (including breast, lung, prostate and colon) a desmoplastic response has been observed, involving activation of fibroblasts in the area and leading to an increased deposition of type I collagen and increased expression of α-SMA (Tlsty & Hein 2001). However, other studies have shown that type I collagen levels are decreased in later stages of tumourigenesis (Fenhalls et al. 1999; van Kempen et al. 2005). Variations in factors such as the stage of tumour development and the region of the tumour tissue that was examined may contribute to the discrepancies in results.

Previous findings suggest that levels of type I collagen present in tumour tissue may differ depending on the stage of tumour progression. In a study performed in our laboratory (Fenhalls et al. 1999) an up-regulation of type I collagen gene expression was observed in stage I breast tumours, while type II and III tumour tissue showed lower levels of type I collagen gene expression. In a study on melanoma tissue, type I collagen gene expression was also shown to be lower in the deeply invasive vertical growth phase of melanomas in the reticular dermis when compared to levels observed in the earlier stages in the papillary dermis (van Kempen et al. 2005). Increased MMP expression was also detected in both fibroblasts and tumour cells in later stage melanomas. Therefore, the decreased levels of

type I collagen protein observed in the tumour tissue could be a result of both degradation by collagenases in the ECM and decreased type I collagen gene expression by fibroblasts in later stage tumours.

Different regions of the tumour may also show variations in type I collagen levels. While the tumour mass itself may have higher levels of type I collagen in the ECM (Bhowmick et al. 2004b), later stage invasive tumours need to overcome the barriers of the surrounding ECM by degrading type I collagen at the invasive front and in the peripheral tumour regions in order to invade the surrounding tissue (Rowe & Weiss 2009). Recent studies have shown that fibroblasts and tumour cells at the invasive front can form migration tracks to facilitate further tumour cell migration (Gaggioli et al. 2007; Wolf et al. 2007). These microtracks are formed in an MMP and adhesion-force-dependent manner, with MT-MMP1 (membrane-type matrix metalloproteinase 1) playing an important role in matrix degradation at the leading invasive edge (Rowe & Weiss 2009). The microtracks facilitate further collective invasion of groups of tumour cells which widen the tracks further to form macrotracks (Wolf et al. 2007). This collective invasion allows tumour cells which have retained their cell-cell junctions to also invade the ECM (Ilina & Friedl 2009).

The presence of certain serum constituents such as urokinase-type plasminogen activator (u-PA) could also play a role in facilitating MMP secretion by tumour cells. Morgan & Hill (2005) found that three different breast cancer cell lines (MDA-MB-231, ZR-75-1 and MCF-7) grown on a type I collagen matrix were capable of degrading the surrounding collagen by secreting MMPs (MMP-1, -3, -9, -13, -14), but only in the presence of u-PA. Interestingly, the urokinase-type plasminogen activator receptor-associated protein (Endo180) was also shown to be involved in the uptake and degradation of type I collagen by tumour cells (Wienke et al. 2007). Under normal conditions this protein is only expressed by fibroblasts, where it functions as a collagen receptor involved in the binding and uptake of extracellular collagens, which are then targeted to intracellular organelles for degradation. It therefore facilitates ECM remodeling and also functions as a promigratory receptor. Endo180 expression was also detected in a subset of basal-like breast tumour

cells and in epithelial tumour cell lines that have a high invasive ability. The expression of this protein by tumour cells was linked to increased tumour growth and reduced tumour collagen content, and was also an independent predictor for shorter disease-free patient survival. Interestingly, mice with a targeted deletion in the Endo180 gene that were crossed with tumour-prone mice had a decreased tumour burden, thought to be a result of the inability of surrounding stromal fibroblast to clear collagens from the invasive front (Madsen et al. 2007; Curino et al. 2005; Wienke et al. 2007). The balance between type I collagen synthesis and degradation is therefore essential in determining the ability of tumour cells to invade the surrounding ECM.

1.4.5 Study hypothesis

This study is based on the hypothesis that tumour cells grown in close proximity to normal fibroblasts have a negative regulatory effect on type I collagen gene expression.

1.4.6 Significance

Current knowledge of the role of tumour cells in regulating the expression of ECM production in neighbouring fibroblasts is limited. Since type I collagen forms the main component of the ECM, a better understanding of the mechanisms involved in tumour-mediated regulation of type I collagen gene expression in fibroblasts could result in a better understanding of the process of tumour invasion and lead to the development of novel drug targets to inhibit tumour invasion and metastasis.

1.4.7 Aims and objectives

The goal of this study was to investigate the effect of breast tumour cells on type I collagen gene expression in neighbouring fibroblasts. An *in vitro* co-culture system was used to investigate the role of cytokines, adhesion molecules and fibroblast signalling pathways in this regulation, with specific objectives being as follows:

1) To investigate the effect of breast tumour cells on type I collagen gene expression and production in fibroblasts.

   Direct and indirect co-culture models will be used to determine how tumour cells regulate type I collagen gene expression in fibroblasts. Fibroblasts will also be transfected with COL1A2 promoter deletion constructs to determine whether tumour cells regulate COL1A2 promoter activity.

2) To identify signalling pathways involved in tumour-mediated down-regulation of type I collagen gene expression.

   Fibroblast/tumour co-culture medium will be screened for the presence of cytokines and/or growth factors that could be involved in negatively regulating type I collagen gene expression. Specific fibroblast signalling pathways that could be involved in tumour-mediated regulation of type I collagen gene expression will also be further investigated.

3) To characterize the effect of tumour cell/fibroblast co-culture on cell adhesion and ECM components.

   An Extracellular Matrix and Adhesion Molecule microarray will be used to determine the effect of tumour cells on ECM and adhesion molecule gene expression in co-cultured fibroblasts. The role of differentially expressed genes in tumour-mediated regulation of type I collagen gene expression will be further investigated.

CHAPTER TWO

BREAST TUMOUR CELL-MEDIATED MODULATION OF TYPE I COLLAGEN PRODUCTION IN FIBROBLASTS

2.1 INTRODUCTION

In normal mammary tissue, epithelial cells form ducts that are separated from the surrounding stroma by a basement membrane. The stroma is made up of fibrillar extracellular matrix (ECM), capillaries and cells such as fibroblasts and immune cells (Kalluri & Zeisberg 2006). The ECM is essential for maintaining tissue structure and homeostasis and loss of this normal tissue architecture has often been associated with cancers (Kenny et al. 2007; Nelson & Bissell 2006).

During the invasive stage of tumour progression, the basement membrane is degraded and tumour cells move into the surrounding stroma, where they come into close contact with stromal cells such as fibroblasts (Kalluri & Zeisberg 2006). This close contact allows for heterotypic interactions to occur between tumour cells and fibroblasts, which would not be possible under normal conditions. Since fibroblasts produce the type I collagen that forms the main component of the ECM, these fibroblast-tumour interactions could lead to changes in type I collagen production by fibroblasts with resultant changes in the tumour ECM.

Previous studies on breast cancer tissue have shown that type I collagen mRNA levels are increased in fibroblasts adjacent to stage I tumours compared to levels in normal breast tissue, but decreased in stage II and stage III tumours. Further *in vitro* studies showed that type I collagen mRNA and protein levels were negatively regulated by direct co-culture of either primary breast tumour cells or WI38 lung fibroblasts with four different breast tumour cell lines, namely; MDA-MB-231, MCF-7, T47D and ZR-75-1 (Fenhalls et al. 1999).

The aim of this chapter was to further investigate the role of breast tumour cells in modulating type I collagen gene expression in normal fibroblasts. Direct and indirect co-culture systems were used to determine the effect of MDA-MB-231 breast tumour cells on type I collagen gene expression in normal WI38 lung fibroblasts and CCD-1068SK breast skin fibroblasts. The MDA-MB-231 breast tumour cell line was chosen, since these cells had previously been shown to significantly repress type I collagen gene expression in normal fibroblasts during direct co-culture experiments (Fenhalls et al. 1999). This tumour cell line was originally derived from a patient with invasive ductal carcinoma (grade III, poorly differentiated) and has been characterised as a basal type breast cancer that is oestrogen and progesterone negative (Lacroix et al. 2004). It therefore represents a highly invasive and relatively aggressive type of breast cancer. Fibroblasts were also co-cultured with MCF12A non-tumourigenic epithelial cells to enable comparison of the effect of non-tumourigenic epithelial versus tumour cells on type I collagen gene expression. The effect of tumour cells on COL1A2 promoter activity was also investigated in WI38 fibroblasts by using COL1A2 promoter deletion constructs. The results of these experiments enabled us to determine the region of the COL1A2 promoter that was involved in tumour-mediated COL1A2 gene regulation.

2.2 RESULTS

2.2.1 The effect of breast tumour cells on type I collagen gene expression after indirect co-cultures with normal fibroblasts.

Tumour cells are able to communicate with fibroblasts in a paracrine manner by secreting soluble factors such as cytokines and growth factors. MDA-MB-231 breast tumour cells may therefore modulate type I collagen gene expression in neighbouring fibroblasts via such secreted factors. To investigate this possibility, an indirect co-culture system was used to separate the fibroblasts from the tumour cells during co-culture.

CCD-1068SK breast skin fibroblasts were indirectly co-cultured with an equal number of MDA-MB-231 tumour cells for 48 hours (section 6.3). A transwell insert with a 0.2 μm pore size was used to allow secreted factors to pass through, but prevent direct contact between fibroblasts and tumour cells. RNA was extracted from CCD-1068SK fibroblasts after indirect co-culture and used for quantitative real-time PCR analysis (section 6.6 to 6.8). Results showed that neither COL1A1 nor COL1A2 mRNA levels were changed in CCD-1068SK fibroblasts indirectly co-cultured with MCF12A epithelial cells, when compared to levels observed in co-cultures with CCD-1068SK fibroblasts (figure 2.1 A). Indirect co-culture with MDA-MB-231 tumour cells also did not affect COL1A2 mRNA levels, while COL1A1 mRNA levels were significantly up-regulated. Protein was also extracted from co-cultured cells and used to determine endogenous α1(I) and α2(I) procollagen levels by means of immunoblotting with a type I collagen antibody that recognises both procollagen chains (section 6.9 - 6.11). Results showed that procollagen levels in CCD-1068SK fibroblasts were not affected by indirect co-culture with either MCF12A or MDA-MB-231 cells (figure 2.1 B).

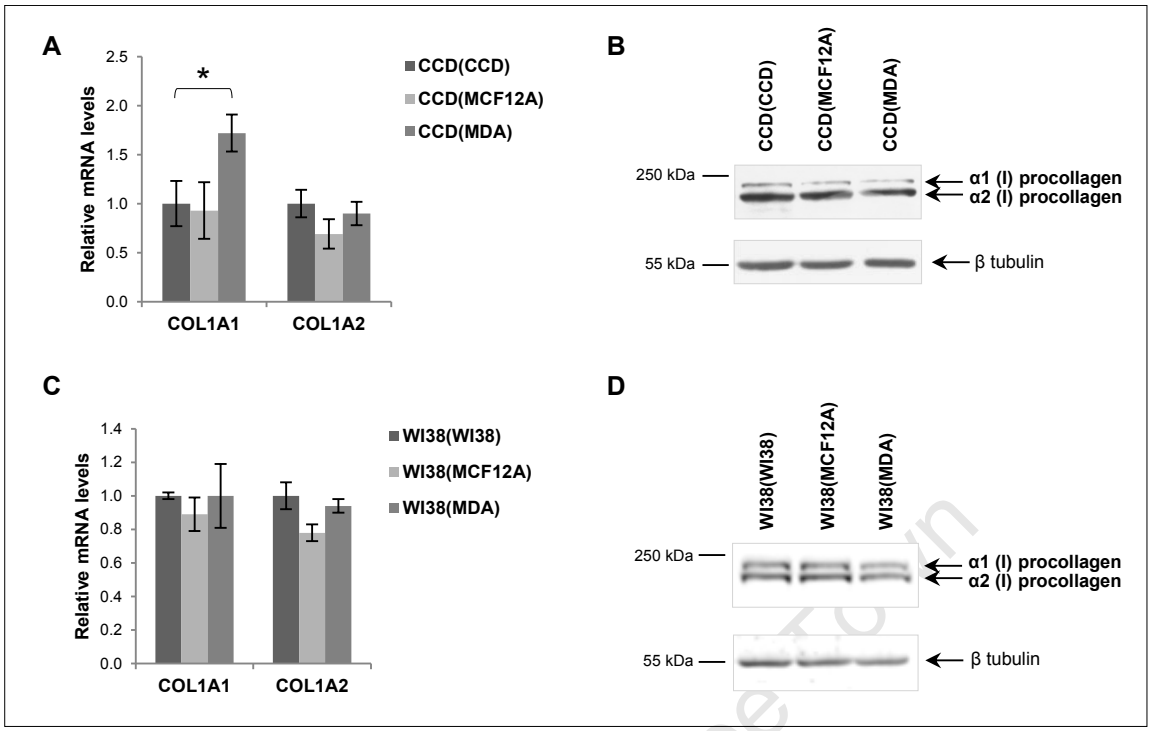

FIGURE 2.1 Type I collagen mRNA and protein levels in normal fibroblasts after indirect co-culture with MDA-MB-231 breast tumour cells. MDA-MB-231 tumour cells were indirectly co-cultured with an equal number of CCD-1068SK or WI38 fibroblasts for 48 hours. (A) Real-time PCR results show relative COL1A1 and COL1A2 mRNA levels in CCD-1068SK fibroblasts after indirect co-culture with MDA-MB-231 tumour cells. Indirect co-cultures of CCD-1068SK fibroblasts with an equal number of MCF12A cells or CCD-1068SK fibroblasts were used as controls. The graph shows the mean ± SD from a representative experiment. (*$p \leq 0.05$, $n=3$). (B) Immunoblotting results show endogenous α1(I) and α2(I) procollagen levels in CCD-1068SK fibroblasts after indirect co-culture with tumour cells. β-tubulin was used as a loading control. (C) Real-time PCR analysis of COL1A1 and COL1A2 mRNA levels in WI38 fibroblasts indirectly co-cultured with tumour cells. (D) Immunoblotting analysis of endogenous α1(I) and α2(I) procollagen levels in WI38 fibroblasts after indirect co-culture. Abbreviations: CCD, CCD-1068SK; MDA, MDA-MB-231. Brackets enclose the cell line with which CCD-1068SK or WI38 fibroblasts were indirectly co-cultured.

WI38 lung fibroblasts were also indirectly co-cultured with MDA-MB-231 tumour cells in a similar manner to CCD-1068SK fibroblasts. Real-time PCR results showed that COL1A1 and COL1A2 mRNA levels in WI38 fibroblasts were not changed after indirect co-culture with MCF12A or MDA-MB-231 cells (figure 2.1 C). Levels of α1(I) and α2(I) procollagen were also not affected by indirect co-culture of WI38 fibroblasts with either MCF21A or MDA-MB-231 cells (figure 2.1 D).

Regulation of type I collagen gene expression in both CCD-1068SK and WI38 fibroblasts was therefore not significantly influenced by factors secreted from either MCF12A epithelial cells or MDA-MB-231 tumour cells when cell types were separated from each other. Although COL1A1 mRNA levels were increased in CCD-1068SK fibroblasts

indirectly co-cultured with MDA-MB-231 tumour cells, COL1A2 mRNA and type I procollagen levels remained unchanged.

2.2.2 The effect of breast tumour cells on type I collagen gene expression in direct co-cultures with fibroblasts.

To determine the effect of breast tumour cells on type I collagen gene expression when in close contact with fibroblasts, a direct co-culture system was employed. This involved mixing MDA-MB-231 tumour cells with an equal number of normal fibroblasts for 48 hours (section 6.2).

After direct co-culture of CCD-1068SK fibroblasts with MDA-MB-231 or MCF12A cells, RNA and protein was extracted from mixed cells and used for further analysis. Since MDA-MB-231 and MCF12A cells do not express type I collagen, results obtained are only a reflection of type I collagen production by fibroblasts. Real-time PCR analysis of RNA isolated from CCD-1068SK/MDA-MB-231 co-cultures showed that both COL1A1 and COL1A2 mRNA levels were significantly down-regulated ($p \leq 0.01$) when compared to levels in CCD-1068SK monocultures. No change in COL1A1 or COL1A2 mRNA levels was observed in the control CCD-1068SK/MCF12A co-cultures (figure 2.2 A). Levels of endogenous α1(I) and α2(I) procollagen were also lower in CCD-1068SK fibroblasts directly co-cultured with MDA-MB-231 cells, but remained unchanged in co-cultures with MCF12A cells (figure 2.2 B).

A radioactive-based assay was used to independently determine the level of secreted α1(I) and α2(I) procollagen protein synthesised by CCD-1068SK fibroblasts during co-cultures. Briefly, [^{3}H]-proline was added to CCD-1068SK/MDA-MB-231 co-culture medium during incubation, which allowed for its incorporation into α1(I) and α2(I) procollagen. Type I collagen cross-linking was prevented by adding β-aminopropionitrile to the medium. After 20 hours incubation the medium was removed from cells and the samples, containing secreted procollagen, were processed as described in Materials and Methods (section 6.12). The results showed that lower levels of α1(I) and α2(I) procollagen were secreted into the medium of CCD-1068SK fibroblasts co-cultured with MDA-MB-231

tumour cells, when compared to levels in CCD-1068SK monocultures and CCD-1068SK/MCF12A co-cultures (figure 2.2 C). The results of the [^{3}H]-proline assay also confirmed that the α1(I) and α2(I) procollagen polypeptides were being synthesized in the correct 2:1 ratio. This ratio was not observed in immunoblotting assays due to differences in specificity of the type I collagen antibody for α2(I) and α1(I) procollagen.

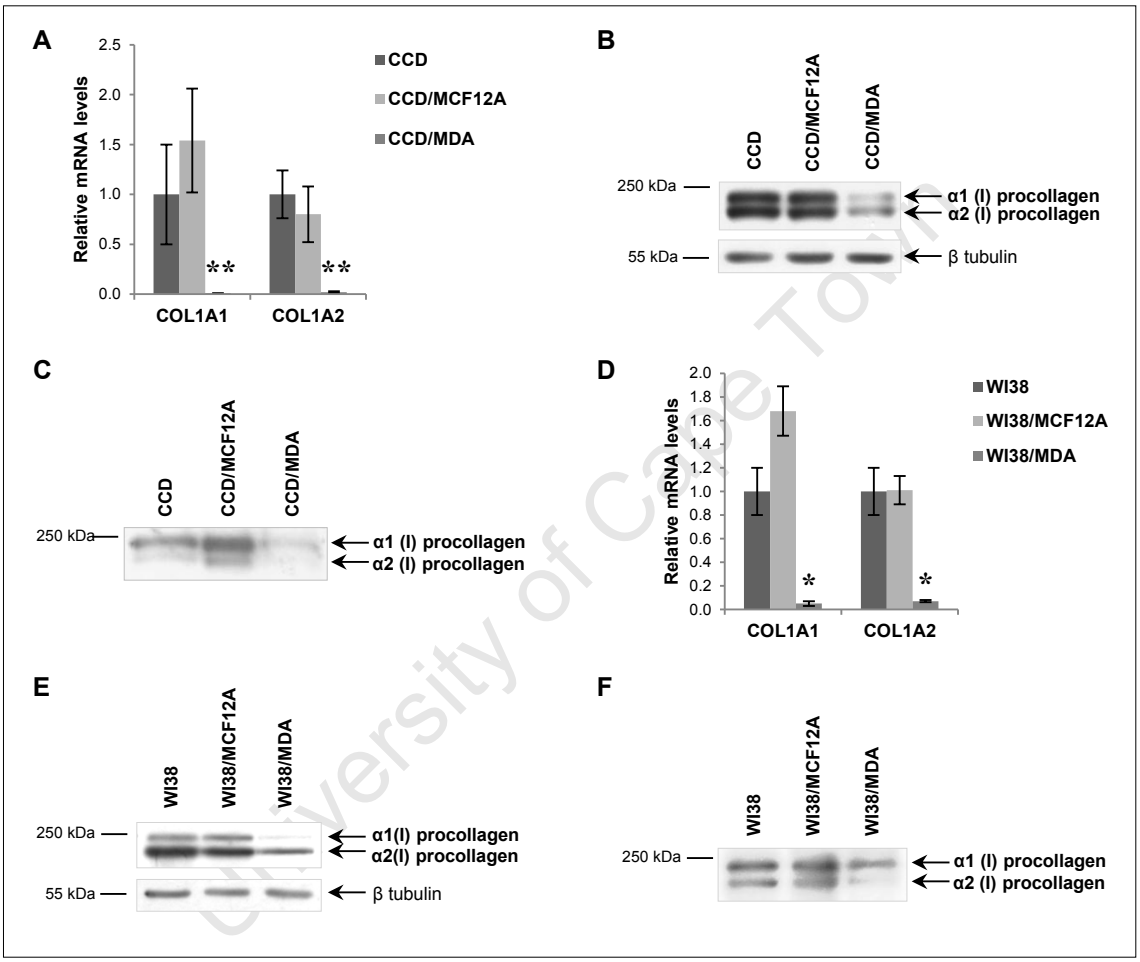

FIGURE 2.2 Type I collagen mRNA and protein levels in normal fibroblasts after direct co-culture with MDA-MB-231 breast tumour cells. MDA-MB-231 tumour cells were directly co-cultured with an equal number of fibroblasts for 48 hours. Fibroblast monocultures and co-cultures with MCF12A cells were used as controls. (A) Real-time PCR analysis of COL1A1 and COL1A2 mRNA levels in CCD-1068SK/MDA-MB-231 co-cultures. The graph shows the mean ± SD from a representative experiment (** $p \leq 0.01$, $n=3$). (B) Immunoblotting results show endogenous α1(I) and α2(I) procollagen protein levels in CCD-1068SK fibroblasts after direct co-cultures. (C) *De novo* synthesis of type I collagen measured by [^{3}H]-proline incorporation into CCD-1068SK/MDA-MB-231 co-culture medium. (D) Real-time PCR analysis of COL1A1 and COL1A2 mRNA levels in WI38/MDA-MB-231 co-cultures. The graphs shows the mean ± SD. (* $p \leq 0.05$, $n=3$). (E**)** Immunoblotting results show endogenous α1(I) and α2(I) procollagen protein levels in WI38/MDA-MB-231 co-cultures. (F) *De novo* synthesis of type I collagen in WI38/MDA-MB-231 co-culture medium.

Direct co-culture of MDA-MB-231 tumour cells with WI38 lung fibroblasts also resulted in a decrease in both COL1A1 and COL1A2 mRNA levels in fibroblast, relative to WI38 monocultures and co-cultures with MCF12A cells (figure 2.2 D). Close contact with MDA-MB-231 tumour cells also decreased endogenous α1(I) and α2(I) procollagen levels (figure 2.2 E) and *de novo* synthesis of type I procollagen (figure 2.2 F). MCF12A co-culture did not significantly influence type I procollagen levels in WI38 fibroblasts.

These results show that MDA-MB-231 tumour cells require close contact with the normal CCD-1068SK and WI38 fibroblasts in order to down-regulate type I collagen gene expression. This regulation occurs at the mRNA level with a resulting decrease in endogenous α1(I) and α2(I) procollagen levels as well as the amount of secreted procollagen polypeptides. No change in type I collagen gene expression was observed in direct co-cultures with MCF12A cells, and therefore the negative regulatory effect on type I collagen synthesis is specifically as a result of the presence of tumour cell.

2.2.3 Separation of normal breast skin fibroblasts after direct co-culture with breast tumour cells.

Thus far, analysis of type I collagen gene expression involved the extraction of total RNA and protein from the combined CCD-1068SK/MDA-MB-231 cell populations after direct co-culture. Although type I collagen was only produced by fibroblasts, the housekeeping genes and proteins used to normalize the results were produced by both cell types. To more accurately determine type I collagen mRNA and procollagen levels in fibroblasts after co-culture, CCD-1068SK fibroblasts were separated from MDA-MB-231 tumour cells again after direct co-culture.

CCD-1068SK fibroblasts were labelled with PKH67 green fluorescent dye before direct co-culture with MDA-MB-231 cells and, after 48 hours co-culture, the two cell types were separated by means of fluorescence-activated cell sorting (FACS) (section 6.5). According to post-sort analysis, the separated populations of CCD-1068SK fibroblast and MDA-MB-231 cells were 97 % and 99.5 % pure, respectively (figure 2.3 A & B). Although the purity of separated populations was high, the number of successfully sorted cells was quite

low, with an average of 20% of the original population being collected. This low number could be due to factors such as cell clumping, low fluorescent intensity or high flow rate. However, further attempts to optimize these parameters did not result in any major increase in the efficiency of cell recovery.

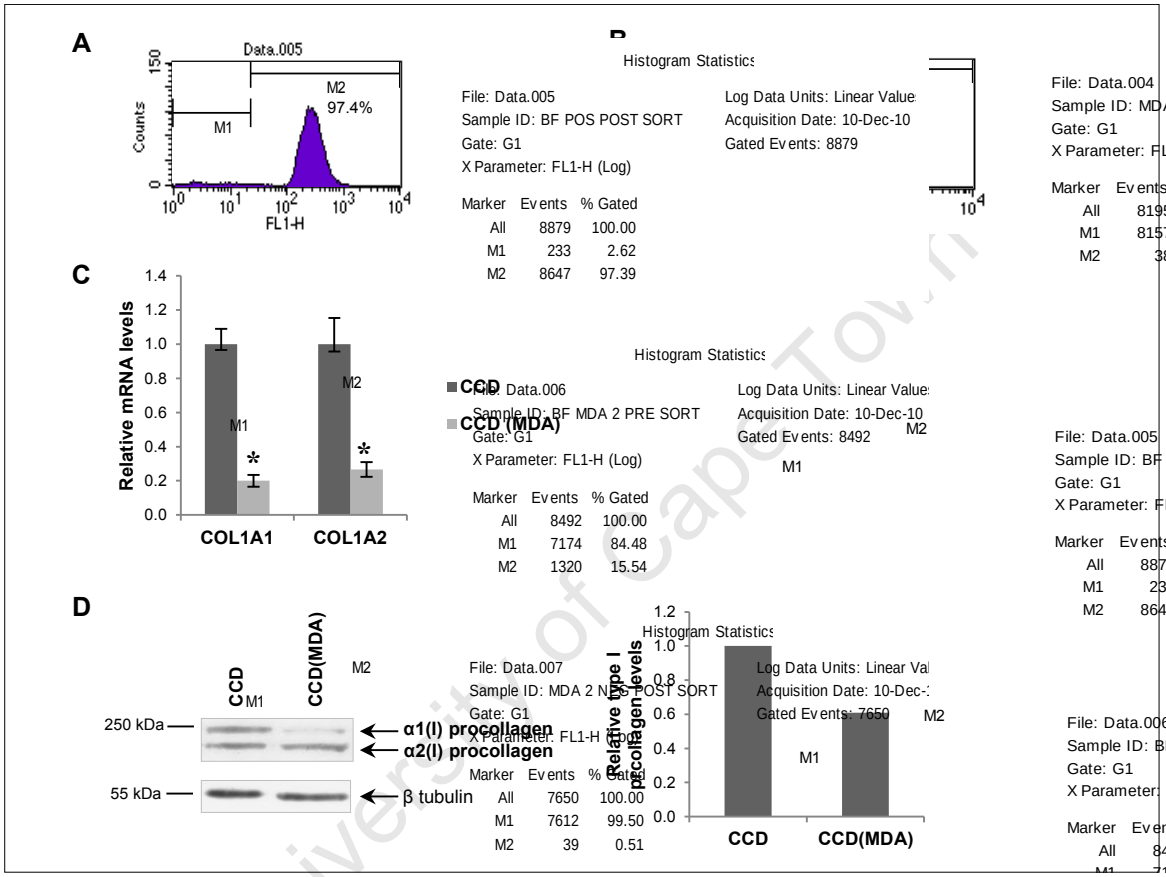

FIGURE 2.3 Type I collagen mRNA and protein levels in CCD-1068SK fibroblasts after direct co-culture with tumour cells and separation of cell types by means of FACS. CCD-1068SK fibroblasts were labeled with PKH67 green fluorescent dye and directly co-cultured with an equal number of MDA-MB-231 tumour cells for 48 hours. FACS was used to separate the fibroblasts and tumour cell populations after co-culture. (A) Post-sort analysis of CCD-1068SK fibroblast population purity. (B) Post-sort analysis of MDA-MB-231 cell population purity. (C) Real-time PCR results show COL1A1 and COL1A2 mRNA levels in CCD-1068SK fibroblasts after co-culture with MDA-MB-231 cells. The graph shows the mean ± SD from a representative experiment (* $p \leq 0.05$, $n=3$). (D) Immunoblotting results show endogenous type I procollagen levels in CCD-1068SK fibroblasts after direct co-culture with MDA-MB-231 tumour cells. Type I procollagen bands were quantified by densitometry analysis and averaged against β-tubulin values to determine relative type I procollagen levels.

Real-time PCR analysis of COL1A1 and COL1A2 mRNA levels in separated CCD-1068SK fibroblasts showed that direct co-culture with MDA-MB-231 tumour cells caused

a significant down-regulation in type I collagen mRNA levels as well as a decrease in procollagen levels (figure 2.3 C & D), and therefore confirm our previous findings.

2.2.4 Temporal regulation of type I collagen gene expression in normal breast skin fibroblasts after direct co-culture with breast tumour cells.

Thus far, type I collagen mRNA and protein levels appear to be down-regulated in CCD-1068SK and WI38 fibroblasts after 48 hours direct co-culture with MDA-MB-231 cells. We therefore wanted to further investigate at what point this negative regulation started and for how long this effect lasted. To determine these parameters, CCD-1068SK fibroblasts were directly co-cultured with MDA-MB-231 cells as previously described, with RNA and protein being extracted from cells at varying time points ranging between 4 hours and 6 days.

Analysis of COL1A1 and COL1A2 mRNA levels showed that MDA-MB-231 tumour-mediated down-regulation of type I collagen gene expression in fibroblasts occurred as early as four hours after changing medium on co-cultured cells, with a more substantial decrease observed after 24 hours (figure 2.4 A). Type I procollagen levels were also only noticeably decreased after 24 hours (figure 2.4 B). This discrepancy between mRNA and protein levels at four and eight hours could be a result of delayed protein processing. Type I procollagen polypeptides undergo extensive post-translational modification in the ER and Golgi before being secreted from the cell. Therefore, endogenous type I procollagen processed before co-culture could still have been present in the fibroblasts four and eight hours after addition of tumour cells. Both type I collagen mRNA and protein levels were however still significantly decreased for up to 6 days after CCD-1068SK/MDA-MB-231 co-culture.

These results suggest that MDA-MB-231 tumour-mediated down-regulation of type I collagen mRNA levels in CCD-1068SK fibroblasts occurs at an early time point, with a delayed response at the protein level, possibly due to extensive procollagen processing. Since the negative modulatory effect of tumour cells on both type I collagen mRNA and protein levels was observed up to 6 days later, this effect is most likely not a result of transiently produced tumour factors.

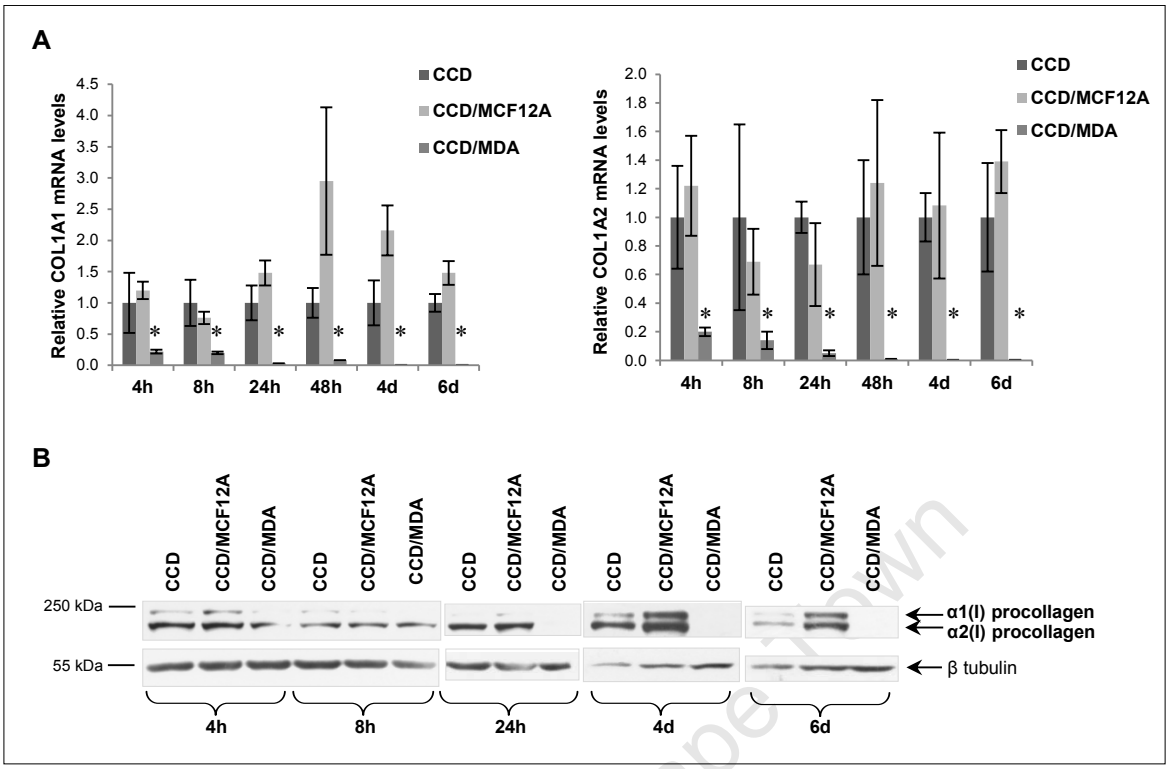

FIGURE 2.4 Temporal analysis of type I collagen mRNA and protein levels in fibroblast/tumour direct co-cultures. CCD-1068SK fibroblasts were directly co-cultured with an equal number of MDA-MB-231 tumour cells and allowed to settle overnight. Medium was then changed and RNA and protein was isolated from cells at different time points (4h, 8h, 24h, 4 days and 6 days) for further analysis. Fibroblast monocultures and co-cultures with MCF12A epithelial cells were used as controls. (A) Real-time PCR results show COL1A1 and COL1A2 mRNA levels in CCD-1068SK/MDA-MB-231 co-cultures. The graph shows the mean ± SD from a representative experiment (*p≤0.05, *n*=3). (B) Immunoblotting results show endogenous α1 and α2(I) procollagen levels in CCD-1068SK fibroblasts after direct co-culture with MDA-MB-231 tumour cells.

2.2.5 COL1A2 promoter activity in normal WI38 lung fibroblasts directly co-cultured with breast tumour cells.

Since tumour cells negatively regulate type I procollagen mRNA levels in neighbouring WI38 and CCD-1068SK fibroblasts, regulation may be occurring at the transcriptional level as a result of decreased COL1A1 and COL1A2 promoter activity. We therefore investigated whether MDA-MB-231 tumour cells negatively regulate COL1A2 promoter activity in fibroblasts during direct co-cultures and, if so, which region(s) of the promoter are involved.

Since both WI38 and CCD-1068SK fibroblasts responded to tumour cell co-culture in a similar manner and, since the transfection efficiency for CCD-1068SK fibroblasts was very poor, COL1A2 promoter studies were performed in WI38 lung fibroblasts. Three COL1A2 promoter deletion constructs were used for COL1A2 promoter assays, each

containing a luciferase gene driven by either the -700/+54, -375/+54, or -107/+54 region of the COL1A2 promoter. WI38 lung fibroblasts were co-transfected with promoter constructs and a CMV-βgal plasmid, which was used as an internal control. Transfected WI38 fibroblasts were then co-cultured with an equal number of MDA-MB-231 or MCF12A cells for 48 hours. Reporter assays were then performed as described in Materials and Methods (section 6.13).

The three COL1A2 promoter deletion constructs had varying basal promoter activity when expressed in WI38 fibroblasts (figure 2.5). The -375/+54 and -700/+54 promoter constructs had similar basal activity, while the activity of the -107/+54 was lower. COL1A2 promoter activity was significantly down-regulated in all three constructs upon co-culture of WI38 fibroblasts with MDA-MB-231 tumour cells.

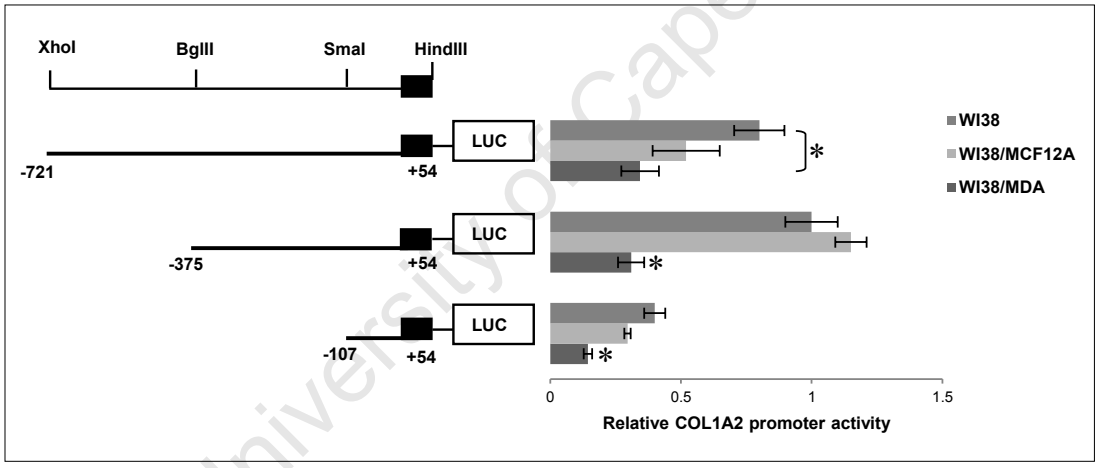

FIGURE 2.5 COL1A2 promoter activity in WI38 lung fibroblasts directly co-cultured with tumour cells. WI38 fibroblasts were co-transfected with one of three minimal COL1A2 promoter deletion constructs and pCMV-βgal. These cells were then co-cultured with MDA-MB-231 tumour cells for 48 hours. WI38 monocultures and co-cultures with MCF12A cells were used as controls. Results of luciferase assays show promoter activity for the -721/+54, -375/+54, and -107/+54 COL1A2 promoter region. Luciferase values were normalized against β-galactosidase values. The graph shows the mean ± SD from a representative experiment (*$p \leq 0.05$, $n=3$)

These results suggest that MDA-MB-231 tumour cells negatively regulate COL1A2 promoter activity, with specific involvement of the -375/+54 region of the COL1A2 promoter. Although the activity of the -107/+54 region was also decreased after tumour cell co-culture, the low basal activity of this region of the promoter makes it difficult to discern its true role in tumour-mediated down-regulation of type I collagen gene expression.

2.3 DISCUSSION

The results in this chapter suggest that close contact between MDA-MB-231 breast tumour cells and normal breast skin or lung fibroblasts results in a significant down-regulation of type I collagen gene expression as well as protein secretion from fibroblasts into the medium. Tumour-mediated suppression of COL1A2 gene transcription was shown to occur at the promoter level, with specific involvement of the -375/+54 region of the COL1A2 promoter. Since no change in type I collagen gene expression was observed in normal fibroblasts co-cultured with non-tumourigenic epithelial cells, negative regulation of type I collagen appears to be tumour-specific and would therefore not occur under normal conditions.

The fact that MDA-MB-231 tumour cells were only able to down-regulate type I collagen gene expression when in close contact with normal fibroblasts suggests that this regulation may well occur during the invasive stages of breast cancer, when tumour cells are in close contact with surrounding fibroblasts as a result of basement membrane degradation. These results support those reported using breast tumour tissue (Fenhalls et al. 1999) and melanoma tissue (van Kempen et al. 2005), showing that levels of type I collagen gene expression were only decreased in later stages of tumour progression.

Although previous work by Fenhalls et al. (1999) also showed that direct co-culture of normal breast skin and lung fibroblasts with MDA-MB-231 breast tumour cells resulted in a decrease in type I collagen gene expression, our study has expanded on these results by using more sensitive techniques such as real-time PCR to analyse COL1A1 and COL1A2 mRNA levels and immunoblotting analysis to analyse endogenous type I procollagen levels. Our study was also the first to show that the presence of tumour cells during analysis of type I collagen gene expression was not skewing the results. This was done by using FACS to separate the fibroblasts from the tumour cells before further analysis of type I collagen mRNA and protein levels in fibroblasts only. Addition of tumour-conditioned medium to fibroblasts by Fenhalls et al. (2009) did not result in any change in type I collagen gene expression, and we observed similar results in our indirect co-culture experiments. However, the results of our model also suggest that factors secreted by

fibroblasts into the medium do not stimulate tumour cells to produce a factor which would, in turn, negatively regulate type I collagen gene expression.

MDA-MB-231 breast tumour cells negatively regulated type I collagen gene expression in both normal breast skin and lung fibroblasts in a similar manner, which could be a result of inherent similarities between the breast and lung tissue environments. The "seed and soil" hypothesis, originally proposed by Stephen Paget (Paget 1889, reprinted as Paget, 1989), states that tumour cells ("seeds") that have moved from the primary tumour into the vasculature will only metastasize to organs with a compatible tissue environment ("soil"). This model has become widely accepted as a way of explaining why certain tumours metastasize to specific organs with higher frequency (Lu & Kang 2007) and could therefore explain why breast tumour cells often metastasize to the lung. It also suggests that MDA-MB-231 breast tumour cells may regulate type I collagen gene expression in a similar manner in primary breast tissue and secondary lung metastatic sites.

A number of previous studies performed on invasive breast tumour tissue have observed the presence of activated fibroblasts in and around the invasive tumour mass (Kunz-Schughart & Knuechel 2002a; Rønnov-Jessen et al. 1996). These so called carcinoma associated fibroblasts (CAFs) have an abnormal myofibroblast-like phenotype and are characterised by increased production of α-SMA and ECM components such as type I collagen (Kunz-Schughart & Knuechel 2002a; Kunz-schughart et al. 2003; De Wever et al. 2008). Since our results showed that type I collagen gene expression was reduced in fibroblasts in close contact with tumour cells, it is unlikely that myofibroblast activation occurred in these co-cultured fibroblasts. Although some studies have shown that normal fibroblast are activated when co-cultured with breast tumour cells (Kojima et al. 2010; Rønnov-Jessen et al. 1995), Kunz-Schughart et al. (2001) showed that breast tumour spheroids co-cultured with normal skin fibroblast spheroids did not induce α-SMA expression in the fibroblasts, even though the tumour cells were seen to migrate into the fibroblast spheroids. These results suggest that myofibroblast differentiation may occur independently of tumour invasion and migration. The exact origin of CAFs is also still being debated, and other cell types such as smooth muscle cells, pericytes and bone-

derived stem cells could form part of the CAF population found in tumour tissue (Rønnov-Jessen & Bissell 2008; Hinz et al. 2007).

Further characterisation of tumour-mediated type I collagen gene expression in our study revealed that negative regulation of type I collagen mRNA levels in normal fibroblasts occurred as early as four hours after co-culture, and that both α1(I) and α2(I) procollagen mRNA and protein levels were still repressed up to six days after co-culture. In a previous study by Shephard et al (2004), fibroblasts directly co-cultured with keratinocytes were shown to produce high levels of the cytokine interleukin-1 (IL-1) during the first four days of co-culture, which prevented fibroblasts from being able to respond to TGFβ secreted by keratinocytes until day four. IL-1 levels then dropped, allowing TGFβ to stimulate the differentiation of fibroblasts into myofibroblasts. However, the results of our study suggest that the type I collagen-regulating factor produced by MDA-MB-231 tumour cells is capable of maintaining its effect for an extended period of time.

MDA-MB-231 tumour cells appear to negatively regulate COL1A1 and COL1A2 mRNA levels, α1(I) and α2(I) procollagen levels, as well as the secretion of both procollagen polypeptides from fibroblasts into the medium. Most type I collagen regulation is thought to occur at a transcriptional level and our results show that MDA-MB-231 tumour cells repress COL1A2 promoter activity, with specific involvement of the -375/+54 region. Since this region of the COL1A2 promoter contains a number of different binding sites for various transcription factors that may be involved in tumour-mediated negative regulation of COL1A2 promoter activity, further investigation of upstream signalling pathways could assist in determining which of these factors are involved.

CHAPTER THREE

SIGNALLING PATHWAYS INVOLVED IN TUMOUR CELL-MEDIATED DOWN-REGULATION OF TYPE I COLLAGEN GENE EXPRESSION

3.1 INTRODUCTION

Results described in chapter two of this study indicate that type I collagen gene regulation by tumour cells requires close contact between normal fibroblasts and tumours cells. MDA-MB-231 tumour cells could therefore communicate with the fibroblasts either through cell adhesion molecules and/or through secreted factors that can only mediate their effect on cells in close proximity.

The effect of secreted cytokines on COL1A1 and COL1A2 gene expression has been well studied. However, very little is known about the role of tumour-secreted factors in regulating type I collagen gene expression in neighbouring fibroblasts. Previous studies investigating keratinocyte or T-cell interactions with fibroblasts during co-culture showed that regulation of type I collagen gene expression by secreted factors such as IL-1, TNFα, IFNγ and TGFβ only occurred when these cells were directly co-cultured with fibroblasts (Rezzonico et al. 1998; Shephard et al. 2004), suggesting that the regulation of type I collagen gene expression by these secreted factors could be concentration dependent or require the presence of other cell surface receptors (e.g. integrins).

The expression of both the COL1A1 and COL1A2 genes are regulated by a number of different growth factors and cytokines that bind to fibroblast cell surface receptors and activate specific signalling pathways. TGFβ is a well studied positive regulator of type I collagen gene expression, and exerts its effect via the Smad signalling pathway (Chen et al. 1999; Verrecchia & Mauviel 2004; Verrecchia et al. 2001a). Negative regulators of type I collagen gene expression include cytokines such as TNFα (Inagaki et al. 1995; Kouba et al. 1999; Mori et al. 1996), IFNγ (Higashi et al. 2003; Xu et al. 2004), IL-1 (Mauviel et al. 1991) and IL-10 (Reitamo et al. 1994). We wanted to determine whether cytokines or

growth factors secreted either by MDA-MB-231 tumour cells themselves, or as a result of tumour/fibroblast co-culture, were responsible for negatively regulating type I collagen gene expression. To this end, medium obtained from CCD-1068SK/MDA-MB-231 co-cultures and monocultures was analysed by means of an ELISA to determine the levels of 12 different cytokines and growth factors secreted by these cells.

The role of CCD-1068SK breast skin fibroblast signalling pathways in MDA-MB-231 tumour-mediated regulation of type I collagen gene expression was also further investigated. The JAK/Stat signalling pathway has previously been shown to negatively regulate type I collagen mainly through the action of activated Stat1, which competes with Smad3 for binding to p300/CBP and thereby prevents association with and activation of the COL1A2 promoter (Ghosh et al. 2001; Inagaki et al. 2003). The role of type I interferons and the JAK/Stat signalling pathway in tumour-mediated type I collagen regulation was therefore further investigated.

Other signalling pathways that could play a role in tumour-mediated regulation of type I collagen gene expression include members of the MAPK family. MAPKs are involved in mediating cellular responses to a large number of external stimuli (Dhanasekaran & Johnson 2007). They therefore play an important role in maintaining cellular homeostasis and are often deregulated in cancers (Dhanasekaran & Johnson 2007; Dhillon et al. 2007). The JNK, ERK and p38 MAPK signalling pathways have previously been shown to regulate type I collagen gene expression, although their effect appear to be context dependent (Leask & Abraham 2004; Javelaud & Mauviel 2005). Therefore, the role of these MAPK signalling pathways in MDA-MB-231 tumour-mediated regulation of type I collagen gene expression in CCD-1068SK fibroblasts was further investigated by using inhibitors against JNK, MEK1/2 and p38 MAPK.

3.2 RESULTS

3.2.1 Analysing factors secreted during fibroblast/tumour co-culture.

A number of cytokines and growth factors are known to be involved in negatively regulating type I collagen gene expression. We therfore used the Multi-Analyte Profiler ELISarray kit (SA Biosciences) to quantify the levels of 12 cytokines, chemokines and growth factors in MDA-MB-231/CCD-1068SK co-culture medium. These included the well-known regulators of type I collagen: IFNγ, TNFα, IL-1 and TGFβ as well as IL4, IL6, IL10, IL12 and IL17, MCP1 (monocyte chemoattractant protein 1), MIP1α and MIP1β (macrophage inflammatory protein 1α and 1β).

CCD-1068SK fibroblasts were directly co-cultured with an equal number of MDA-MB-231 tumour cells for 48 hours in serum-free medium and the ELISarray kit was used to profile the levels of the secreted factors in the medium, as described in Materials and Methods (section 6.14). Of the 12 cytokines analysed, only TGFβ, IL6 and MCP1 were detected at levels above background in both CCD-1068SK fibroblasts and MDA-MB-231 tumour cells (figure 3.1). The level of TGFβ produced by MDA-MB-231 cells was two-fold higher than levels observed in CCD-1068SK medium, while levels in the CCD-1068SK/MDA-MB-231 co-culture medium were slightly lower than would be expected if the TGFβ values obtained for the fibroblast and tumour monocultures were averaged. These results suggest that levels of secreted TGFβ are decreased as a result of co-culturing CCD-1068SK fibroblasts with MDA-MB-231 tumour cells. Results also indicate that, although IL6 and MCP1 were secreted by both fibroblasts and tumour cells, levels observed in CCD-1068SK/MDA-MB-231 co-cultures did not appear to change as a result of direct co-culture. Levels of the remaining factors screened for were all below detectable levels in the medium of co-cultures and monocultures of each cell type.

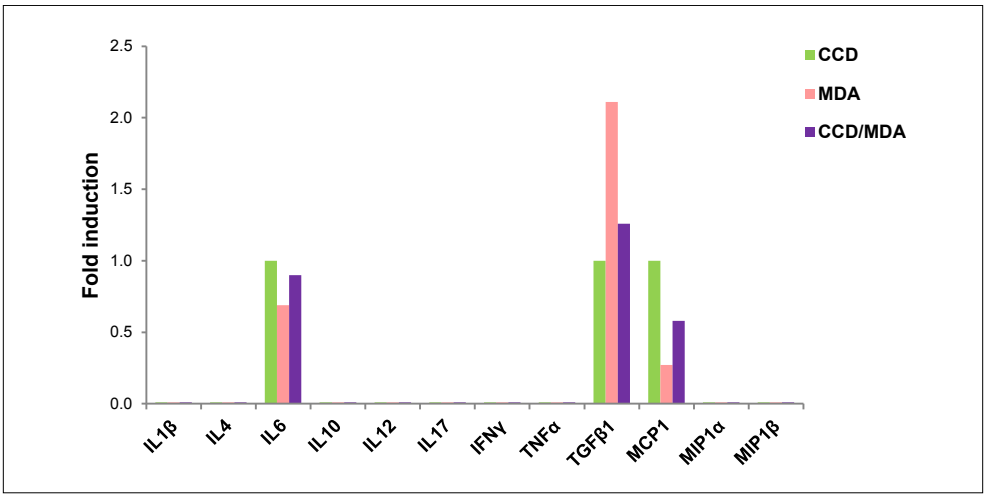

FIGURE 3.1 Cytokine and growth factor production in fibroblast/tumour direct co-cultures. CCD-1068SK fibroblasts were directly co-cultured with MDA-MB-231 tumour cells for 48 hours in serum-free medium. The Multi-Analyte Profiler ELISarray kit (SA Biosciences) was used to quantify levels of 12 different cytokines in the medium of CCD-1068SK/MDA-MB-231 co-cultures, relative to levels obtained in the medium of monocultures of each cell type. These cytokines include IL1b, IL4, IL6, IL10, IL12, IL17a, IFNγ, TNFα, TGFβ, MCP1, MIP1a and MIP1b. Values were normalized to levels observed in the medium of CCD-1068SK monocultures. The graph represents the results of a single experiment with one samples for each assay. Tumour growth factor beta (TGFβ), interleukin 6 (IL6) and monocyte chemoattractant protein 1 (MCP1) were detected in the medium of all three cultures, while levels of the other 9 cytokines were all below background.

These results suggest that none of the negative regulatory factors screened for, including IFNγ, TNFα, IL-1 or IL-10, are secreted by fibroblasts or tumour cells during co-culture or monoculture conditions. These factors are therefore unlikely to be involved in MDA-MB-231 tumour-mediated repression of type I collagen gene expression.

3.2.2 IFNα and IFNβ gene expression in co-cultured cells.

Results of the ELISarray on CCD-1068SK/MDA-MB-231 co-culture medium showed that IFNγ could not be detected in either cell type in monoculture or during direct co-culture. However, this array did not include type I IFNs (IFNα and IFNβ). Since previous studies have shown that both IFNα and IFNβ may negatively regulate type I collagen gene expression (Duncan & Berman 1985; Inagaki et al. 2003), we examined the effect of direct co-culture on the expression of these cytokines in CCD-1068SK fibroblasts and MDA-MB-231 cells.

Since type I IFN production is mainly controlled at the level of gene transcription (Honda et al. 2006), real-time PCR analysis was used to determine IFNα and IFNβ gene expression in CCD-1068SK fibroblasts and MDA-MB-231 cells after co-culture and FACS separation. The results showed that IFNα and IFNβ were expressed by both CCD-1068SK fibroblasts and MDA-MB-231 cells in monocultures. Co-culture with MDA-MB-231 tumour cells resulted in a significant increase in mRNA levels of both IFNα and IFNβ in CCD-1068SK fibroblasts, with IFNβ levels increased more than 40-fold (figure 3.2 A & B). Interestingly, although IFNα mRNA levels remained unchanged in MDA-MB-231 cells after co-culture, IFNβ mRNA levels were significantly up-regulated relative to levels in tumour monocultures.

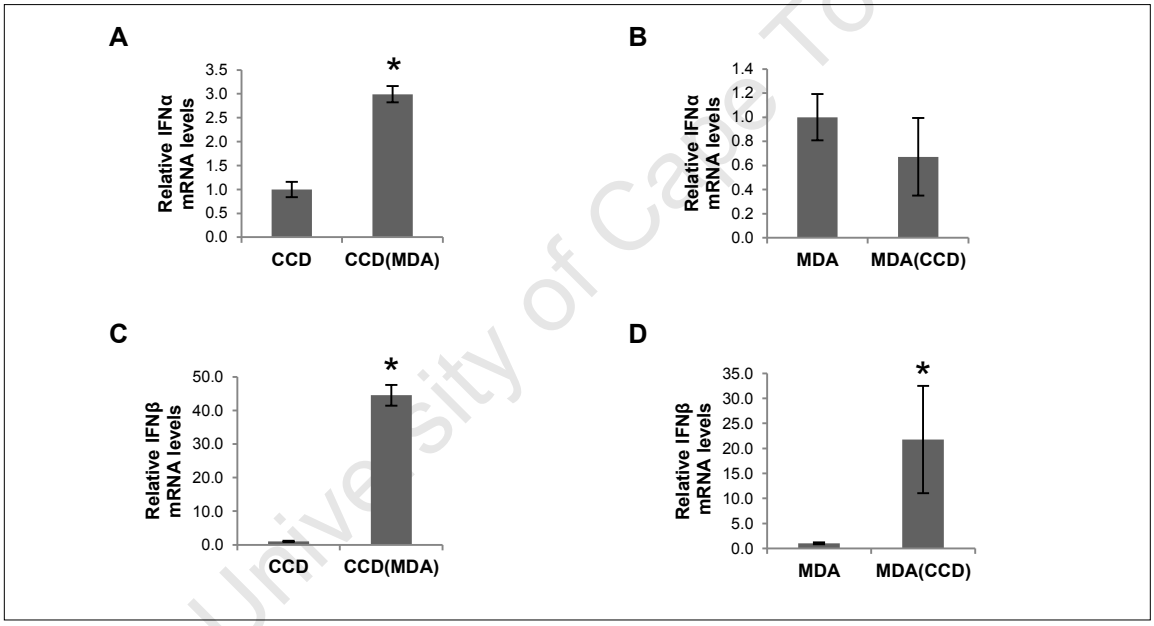

FIGURE 3.2 Type I IFN mRNA levels in directly co-cultured fibroblasts and tumour cells. CCD-1068SK/MDA-MB-231 co-cultures were incubated for 48 hours before separating cell using FACS. Real-time PCR analysis was performed on isolated RNA. (A) Relative interferon alpha (IFNα) mRNA levels in CCD-1068SK fibroblasts after co-culture. (B) Relative IFNα mRNA levels in MDA-MB-231 tumour cells after co-culture. (C) Interferon beta (IFNβ) mRNA levels in co-cultured CCD-1068SK fibroblasts compared to those obtained in monocultures. (D) IFNβ mRNA levels in co-cultured MDA-MB-231 cells compared to those obtained in monocultures. (*$p \leq 0.05$, $n=3$). The graphs shows the mean ± SD from representative experiments. Abbreviations: CCD, CCD-1068SK; MDA, MDA-MB-231; CCD(MDA), CCD-1068SK fibroblasts co-cultured with MDA-MB-231 tumour cells and separated by FACS; MDA(CCD), MDA-MB-231 tumour cells co-cultured with CCD-1068SK fibroblasts and separated by FACS.

These results suggest that direct co-culture of CCD-1068SK fibroblasts with MDA-MB-231 tumour cells causes a significant increase in the expression of IFNα and IFNβ in CCD-

1068SK fibroblasts, and an increase in IFNβ gene expression in MDA-MB-231 tumour cells. Therefore, signalling via type I IFNs could be involved in mediating the negative regulatory effect of MDA-MB-231 tumour cells on type I collagen gene expression.

3.2.3 Stat1 gene expression in co-cultured cells.

Since the type I IFNs were up-regulated in CCD-1068SK fibroblasts and MDA-MB-231 tumour cells during direct co-culture, close contact between these two cell types could be inducing an interferon response. Type I and type II IFNs exert most of their effects via the JAK/Stat signalling pathway, which has previously been shown to negatively regulate type I collagen gene expression in fibroblasts in response to IFN stimulation (Ghosh et al. 2001; Higashi et al. 2003; Inagaki et al. 2003). Since Stat1 is one of the main mediators of this IFN response, the role of Stat1 in mediating the negative regulatory effect of MDA-MB-231 tumour cells on type I collagen gene expression in CCD-1068SK fibroblasts was further investigated.

To determine the effect of co-culture on Stat1 gene expression in fibroblasts and tumour cells, these cells were separated by means of FACS after 48 hours direct co-culture. Real-time PCR results showed that Stat1 mRNA levels were significantly increased in both CCD-1068SK fibroblasts and MDA-MB-231 cells after co-culture (figure 3.3 A & B). Immunoblotting analysis of total Stat1 protein was performed using an anti-Stat1 p84/p91 antibody that detected both the Stat1α (91 kDa) isoform as well as its splice variant Stat1β (84 kDa), which lacks 38 residues at the carboxyl-terminus (Schindler & Darnell 1995). Although only Stat1α was detected in CCD-1068SK fibroblast monocultures, direct co-culture with MDA-MB-231 cells led to an increase in the levels of both the Stat1α and Stat1β isoforms (figure 3.3 C). Both these isoforms were also produced by MDA-MB-231 cells in monoculture, with increased levels of both Stat1α and Stat1β in co-cultures with fibroblasts. Phosphorylation of Stat1 protein at the tyrosine-701 site was also increased in both CCD-1068SK and MDA-MB-231 cells after co-culture, although this increase appeared to be greater in the fibroblasts. Levels of Stat1α phosphorylation were also much higher than those of Stat1β in both fibroblasts and tumour cells.

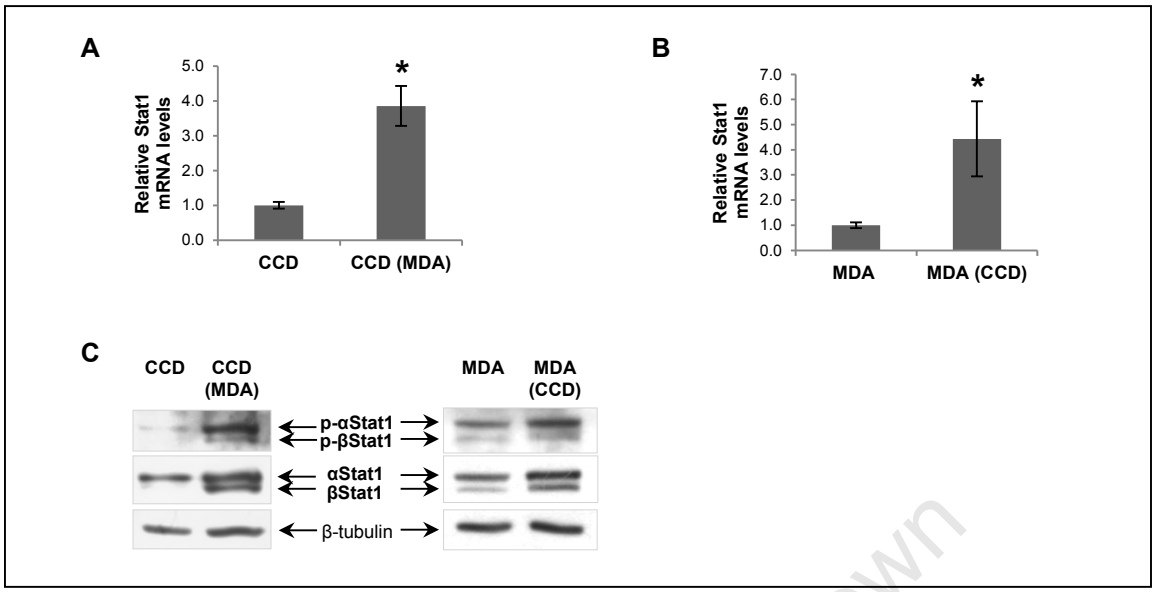

FIGURE 3.3 Stat1 mRNA and protein levels in fibroblast/tumour co-cultures. CCD-1068SK/MDA-MB-231 direct co-cultures were incubated for 48h before being separated again by means of FACS for further analysis. (A) Real-time PCR results show Stat1 mRNA levels in CCD-1068SK fibroblasts after co-culture with MDA-MB-231 tumour cells compared to those obtained in CCD-1068SK monocultures. (B) Stat1 mRNA levels in MDA-MB-231 tumour cells after co-culture with CCD-1068SK fibroblasts, compared to those obtained in MDA-MB-231 monocultures (*$p \leq 0.05$, $n=3$). The graphs shows the mean ± SD from a representative experiment. (C) Immunoblotting analysis of total Stat1 (αStat1 & βStat1) and Tyr-701 phosphorylated Stat1 (p-αStat1 & p-βStat1) in CCD-1068SK fibroblasts and MDA-MB-231 cells after direct co-culture. Abbreviations: CCD, CCD-1068SK; MDA, MDA-MB-231; CCD(MDA), CCD-1068SK fibroblasts co-cultured with MDA-MB-231 tumour cells and separated by FACS; MDA(CCD), MDA-MB-231 tumour cells co-cultured with CCD-1068SK fibroblasts and separated by FACS.

These results show that direct co-culture of CCD-1068SK fibroblasts with MDA-MB-231 tumour cells leads to a significant increase in Stat1 gene expression as well as increased phosphorylation of Stat1 protein in both cell types. Since Stat1 is known to negatively regulate type I collagen gene expression, it is possible that MDA-MB-231 tumour cells suppress type I collagen gene expression by activating the JAK/Stat signalling pathway in neighbouring fibroblasts (figure 1.5).

3.2.4 The effect of inhibiting JAK activation on type I collagen gene expression in co-cultured cells.

Activation of Stat1 occurs via tyrosine phosphorylation mediated by upstream Janus Kinases (JAKs). The four known members of the JAK family are JAK1, JAK2, JAK3 and TYK2, which bind in specific combinations to different cell surface receptors to form dimers. To examine the role of JAK/Stat signalling in mediating the negative regulatory

effect of tumour cells on type I collagen gene expression in fibroblasts, an inhibitor was used to prevent the activation of all members of the JAK family (JAK1, JAK2, JAK3 and Tyk2) by competing with these proteins for binding to ATP.

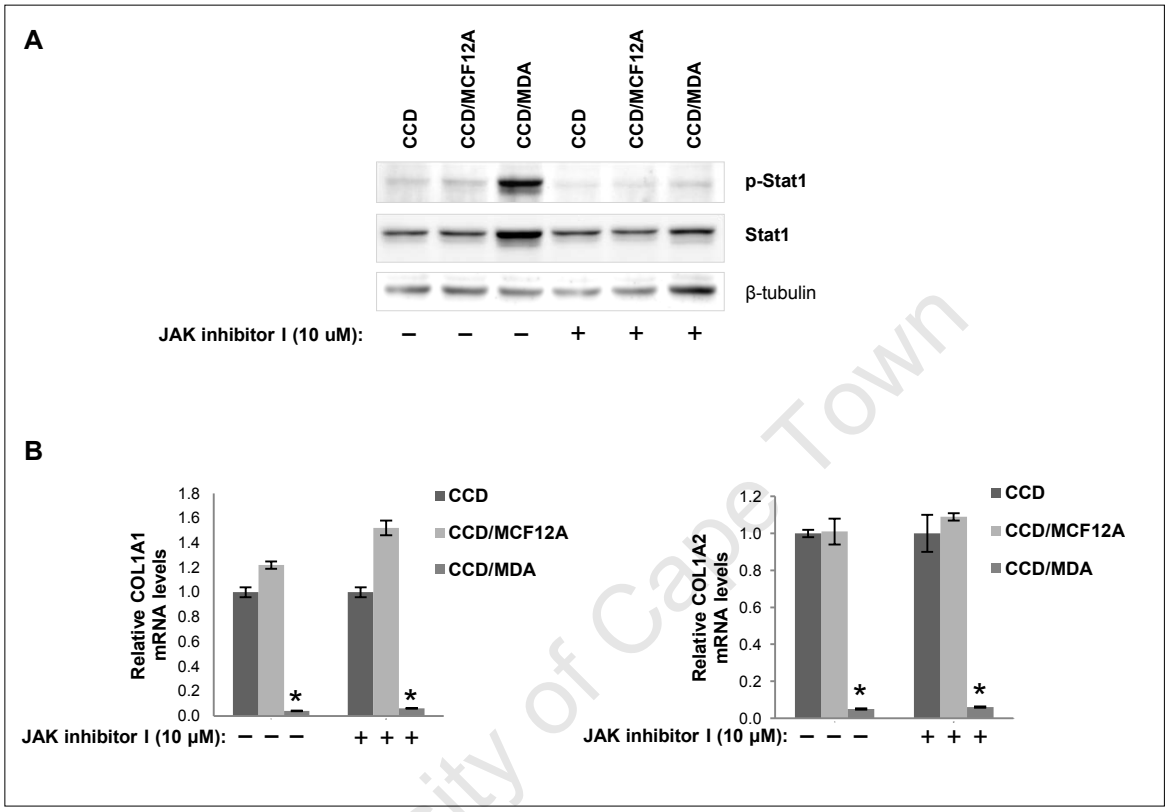

FIGURE 3.4 The effect of inhibiting JAK activation in fibroblast/tumour co-cultures on type I collagen mRNA levels. JAK inhibitor I was added to CCD-1068SK monocultures, as well as CCD-1068SK/MCF12A and CCD-1068SK/MDA-MB-231 co-cultures and incubated for 24 hours. (A) Immunoblotting results show the effect of the JAK inhibitor on Stat1 phosphorylation and total Stat1 protein levels. (B) Real-time PCR results show relative COL1A1 and COL1A2 mRNA levels in co-cultures incubated with JAK inhibitor I. The graphs show the mean ± SD from representative experiments (*$p \leq 0.05$, $n=3$).

CCD-1068SK/MDA-MB-231 co-cultures were incubated with 10 µM JAK inhibitor I for 24 hours (section 6.15). Immunoblotting analysis confirmed that levels of total and phosphorylated Stat1 were only increased in CCD-1068SK/MDA-MB-231 co-cultures, with no change in Stat1 protein levels observed in fibroblast co-cultures with MCF12A cells (figure 3.4 A). Results also showed that Stat1 phosphorylation was inhibited after addition of the JAK inhibitor to CCD-1068SK/MDA-MB-231 co-cultures. Having established that the JAK inhibitor blocked Stat1 phosphorylation in CCD-1068SK/MDA-MB-231 co-cultured cells, we next determined the effect of this inhibition on COL1A1 and

COL1A2 mRNA expression. Real-time PCR analysis revealed that inhibition of JAK signalling in directly co-cultured cells had no effect on COL1A1 or COL1A2 mRNA levels (figure 3.4 B).

These results show that, although direct co-culture of CCD-1068SK fibroblast with MDA-MB-231 tumour cells led to an increase in Stat1 gene expression and protein activation, inhibition of the upstream activators of Stat1 did not reverse the negative regulatory effect of MDA-MB-231 tumour cells on type I collagen gene expression.

3.2.5 The effect of silencing Stat1 in fibroblasts on type I collagen gene expression in co-cultures.

While no change in type I collagen gene expression was observed upon addition of the JAK inhibitor to co-cultured cells, up-regulation of Stat1 gene expression and activation could still occur via a different signalling pathway. Also, since the JAK inhibitor blocked Stat1 phosphorylation in both CCD-1068SK fibroblasts and MDA-MB-231 cells during co-cultures, we could not determine the effect of blocking JAK/Stat signalling in each individual cell type on type I collagen gene regulation. Further experiments therefore involved the selective silencing of Stat1 gene expression in CCD-1068SK fibroblasts before direct co-culture with MDA-MB-231 tumour cells.

CCD-1068SK fibroblasts were transfected with Stat1 siRNA (section 6.16) and then directly co-cultured with non-transfected MDA-MB-231 cells. Quantification of Stat1 mRNA and protein levels in transfected CCD-1068SK fibroblasts confirmed that Stat1 siRNA efficiently silenced Stat1 gene expression, with mRNA levels reduced by more than 90 % (figure 3.5 A & B). Results also showed that type I procollagen levels were not affected by Stat1 silencing in fibroblasts (figure 3.5 B). Analysis of type I collagen mRNA levels in Stat1-inhibited CCD-1068SK fibroblasts after MDA-MB-231 co-culture showed that neither COL1A1 nor COL1A2 mRNA levels were changed, when compared to levels in non-transfected co-cultures (figure 3.5 C).

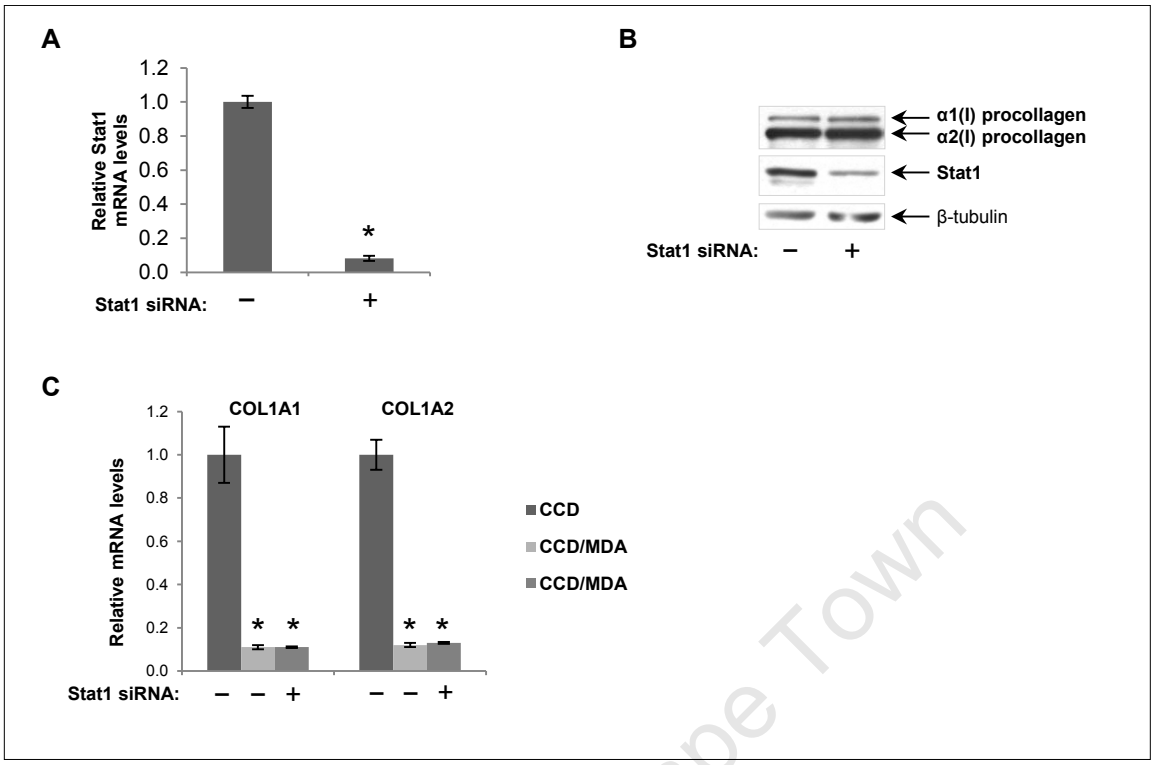

FIGURE 3.5 The effect of silencing Stat1 expression in fibroblasts on type I collagen mRNA levels in co-cultures with tumour cells. CCD-1068SK fibroblasts were transfected with Stat1 siRNA and directly co-cultured with MDA-MB-231 cells for 48 hours. (A) Real-time PCR results show Stat1 mRNA levels in CCD-1068SK fibroblasts after transfection with Stat1 siRNA. (B) Immunoblotting results show total Stat1 protein levels in CCD-1068SK fibroblasts transfected with Stat1 siRNA. (C) COL1A1 and COL1A2 mRNA levels in CCD-1068SK fibroblasts transfected with Stat1 siRNA and directly co-cultured with MDA-MB-231 tumour cells. The graphs show the mean ± SD from representative experiments (*$p \leq 0.05$, $n=3$).

These results show that inhibiting Stat1 gene expression in CCD-1068SK fibroblasts does not revert the negative regulatory effect of MDA-MB-231 on type I collagen gene expression, which suggests that the increased Stat1 gene expression observed in co-cultured CCD-1068SK fibroblasts may not be involved in the tumour-mediated regulation of type I collagen gene expression.

3.2.6 The effect of silencing Stat1 gene expression in tumour cells on type I collagen gene expression in co-cultured fibroblasts.

Since Stat1 expression was also up-regulated in MDA-MB-231 cells after direct co-culture with CCD-1068SK fibroblasts, we examined the effect of inhibiting Stat1 gene expression in MDA-MB-231 tumour cells on type I collagen gene expression in co-cultures.

MDA-MB-231 tumour cells were transfected with Stat1 siRNA and directly co-cultured with CCD-1068SK fibroblasts. Stat1 mRNA and protein levels were successfully silenced in MDA-MB-231 cells, as confirmed by real-time PCR and immunoblotting, respectively (figure 3.6 A & B). However, neither COL1A1 nor COL1A2 mRNA levels were altered in CCD-1068SK fibroblasts co-cultured with Stat1-inhibited MDA-MB-231 cells, when compared to non-transfected co-cultures (figure 3.6 C).

These results show that Stat1 inhibition in tumour cells does not revert the negative regulatory effect of these cells on type I collagen gene expression, and suggests that the increased gene expression and activation of Stat1 observed in co-cultured MDA-MB-231 tumour cells does not play a role in negatively regulating type I collagen gene expression in neighbouring CCD-1068SK fibroblasts.

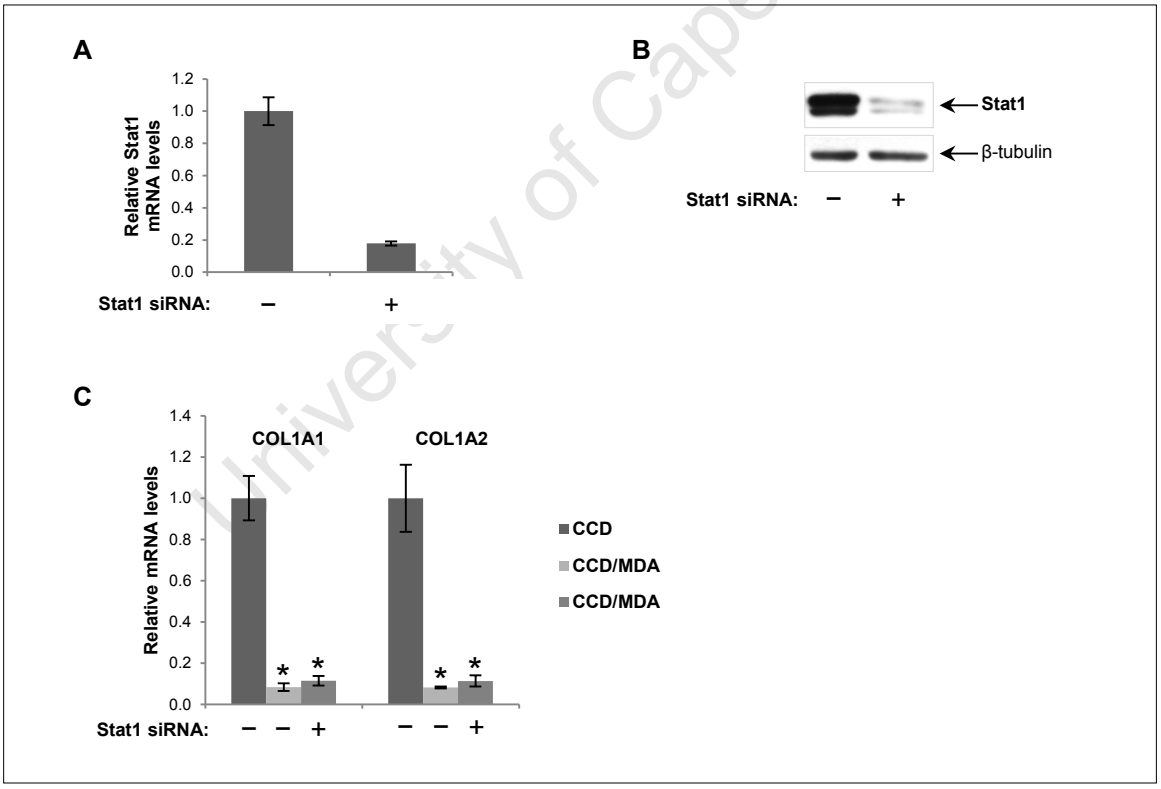

FIGURE 3.6 The effect of silencing Stat1 expression in tumour cells on type I collagen mRNA levels in co-cultured fibroblasts. MDA-MB-231 tumour cells were transfected with Stat1 siRNA and co-cultured with CCD-1068SK fibroblasts for 48 hours. (A) Real-time PCR results show Stat1 mRNA levels in MDA-MB-231 cells transfected with Stat1 siRNA. (B) Immunoblotting results show Stat1 protein levels in MDA-MB-231 cells transfected with Stat1 siRNA. (C) COL1A1 and COL1A2 mRNA levels in CCD-1068SK fibroblasts directly co-cultured with Stat1 siRNA-transfected MDA-MB-231 cells. The graphs show the mean ± SD from representative experiments (*$p \leq 0.05$, $n=3$).

3.2.7 Stat1 gene expression in indirect fibroblast/tumour co-cultures.

Results thus far show that direct co-culturing of CCD-1068SK fibroblasts with MDA-MB-231 tumour cells leads to the up-regulation and possible secretion of type I IFNs into the medium. Binding of these IFNs to IFNα/β receptors on both fibroblasts and tumour cells could, in turn, result in the activation of the JAK/Stat signalling pathway with increased Stat1 phosphorylation, as observed in both cell types. We were interested in determining the exact nature of the IFN response observed during tumour/fibroblast co-culture, and therefore investigated whether Stat1 was being activated in response to factors secreted during CCD-1068SK/MDA-MB-231 direct co-cultures or as a result of factors secreted by MDA-MB-231 cells only.

The indirect co-culturing system was used to determine whether Stat1 and type I collagen gene expression in CCD-1068SK fibroblasts were influenced by secreted factors produced by either MDA-MB-231 cell monocultures or directly co-cultured CCD-1068SK/MDA-MB-231 cells (section 6.3) (figure 3.7 A). This system was previously employed to determine whether MDA-MB-231 tumour cells influenced type I collagen gene expression in CCD-1068SK fibroblasts when separated by a transwell insert (section 2.2.1). No change in type I collagen mRNA or protein levels were observed (figure 2.1), and further analysis showed that Stat1 mRNA levels in CCD-1068SK fibroblasts were also not changed as a result of factors secreted by MDA-MB-231 cells only (figure 3.7 B). However, in CCD-1068SK fibroblasts indirectly co-cultured with mixed CCD-1068SK/MDA-MB-231 cells, Stat1 mRNA levels were increased more than 7-fold. Total Stat1 and phosphorylated Stat1 protein levels in CCD-1068SK fibroblasts were also only increased in indirect co-cultures with mixed fibroblast/tumour cells (figure 3.7 C). However, real-time PCR analysis of COL1A1 and COL1A2 mRNA levels in CCD-1068SK fibroblasts showed that, even when Stat1 activation occurred, type I collagen gene expression remained unchanged after indirect co-culture with mixed CCD-1068SK/MDA-MB-231 cells (figure 3.7 D).

In summary these results show that Stat1 mRNA and protein levels, as well as protein activation, only increased in CCD-1068SK fibroblasts as a result of factors secreted when

fibroblasts were in close contact with MDA-MB-231 tumour cells. However, type I collagen gene expression remained unchanged in CCD-1068SK fibroblasts even when Stat1 activation occurred. These results therefore suggest that the JAK/Stat signalling pathway is not involved in MDA-MB-231 tumour-mediated down-regulation of type I collagen gene expression in CCD-1068SK fibroblasts.

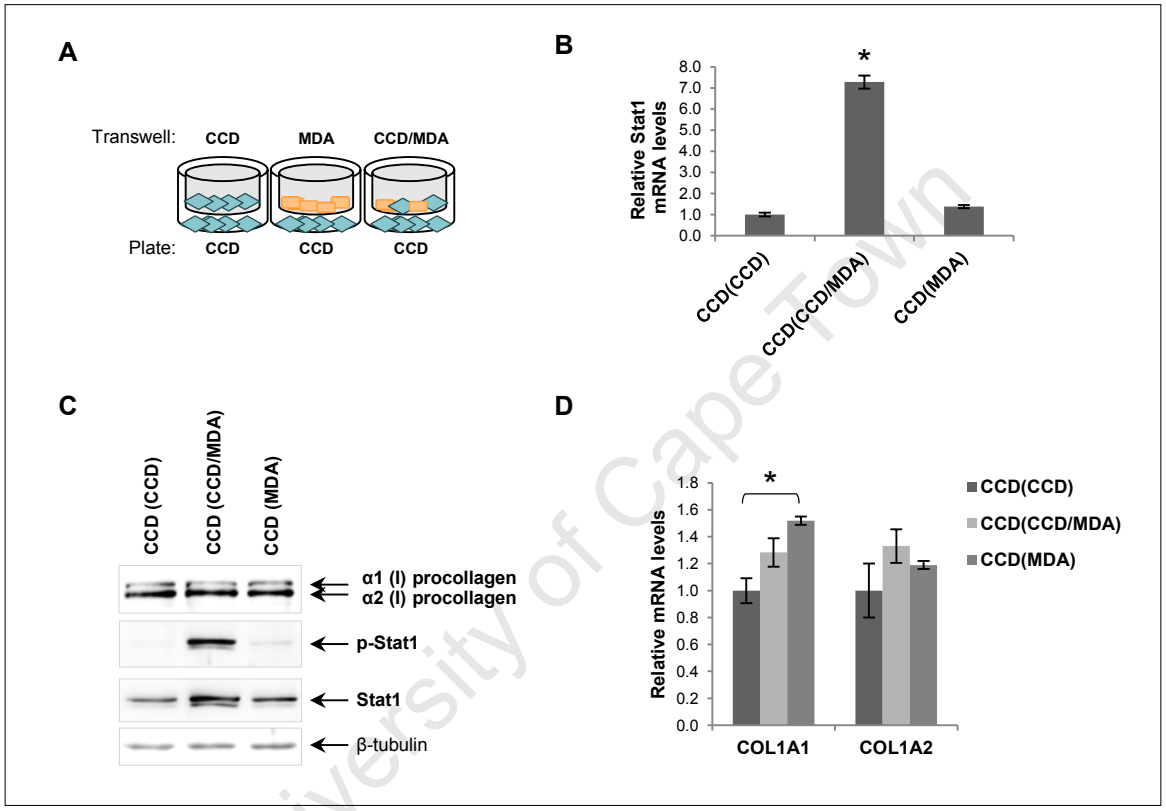

Figure 3.7 The effect of secreted factors produced during co-cultures on Stat1 gene expression in CCD-1068SK fibroblasts. CCD-1068SK fibroblasts were indirectly co-cultured with CCD-1068SK fibroblasts, MDA-MB-231 tumour cells or mixed CCD-1068SK/MDA-MB-231 cells for 48 hours. (A) Diagram representing the transwell co-culture experiment in which CCD-1068SK fibroblasts were separated from an equal number of CCD-1068SK fibroblasts, MDA-MB-231 cells or a mixture of both during co-culture. (B) Stat1 mRNA levels in CCD-1068SK fibroblasts after indirect co-culture with mixed CCD-1068SK/MDA-MB-231 cells. The cells shown in brackets were cultured in the transwell insert. (C) Immunoblotting results show total and phosphorylated Stat1, as well as α1(I) and α2(I) procollagen levels in CCD-1068SK fibroblasts after indirect co-culture. (D) Real-time PCR results show COL1A1 and COL1A2 mRNA levels in CCD-1068SK fibroblasts after indirect co-culture. The graphs show the mean ± SD from representative experiments (*$p \leq 0.05$, $n=3$). Abbreviations: CCD, CCD-1068SK; MDA, MDA-MB-231. Brackets enclose the cell line with which CCD-1068SK fibroblasts were indirectly co-cultured.

3.2.8 MAPK signalling in fibroblast/tumour co-cultures.

MAP kinases are a group of intracellular signalling molecules that play an important role in controlling cellular responses such as cell growth, differentiation and apoptosis (Bardwell 2006). Deregulation of these signalling pathways is therefore often associated with tumour formation and progression. Tumour cells may also deregulate MAPK signalling pathways in neighbouring fibroblasts by means of paracrine signalling and, since these signalling pathways have previously been shown to influence type I collagen gene expression, the role of the JNK, MEK/ERK and p38 MAPK signalling pathways in mediating the negative regulatory effect of tumour cells on type I collagen gene expression in CCD-1068SK fibroblasts was further investigated.

3.2.8.1 JNK signalling in co-cultures.

JNK MAPKs are activated when cells are exposed to specific cytokines, growth factors, and environmental stresses such as UV irradiation and heat shock (Davis 2000). The pro-inflammatory cytokine TNFα is known to antagonise TGFβ-mediated COL1A2 promoter activity by activating JNK signalling with downstream phosphorylation of cJun (and in some cases JunB) which, in turn, binds to Smad3 and inhibits TGFβ/Smad-mediated stimulation of COL1A2 promoter activity (Verrecchia et al., 2003). Although no TNFα was secreted during CCD-1068SK/MDA-MB-231 co-cultures, negative regulation of type I collagen gene expression by the JNK pathway could be mediated via other mechanisms (figure 3.1).

The role of JNK signalling in MDA-MB-231 tumour-mediated type I collagen gene regulation in CCD-1068SK fibroblasts was examined by using SP600125, an inhibitor that prevents JNK (JNK-1, -2 and -3) phosphorylation by competing with JNK for ATP. CCD-1068SK/MDA-MB-231 co-cultures were incubated with SP600125 for 24 hours before isolating RNA and protein from co-cultured cells for further analysis (section 6.16). Immunoblotting results showed that the level of Serine-63/73 phosphorylated cJun was very high in CCD-1068SK/MDA-MB-231 co-cultures compared to the level in CCD-1068SK monocultures or CCD-1068SK/MCF12A co-cultures (figure 3.8 A). Total cJun levels, however, were similar in all cultures. Addition of SP600125 to cells resulted in a

significant reduction in levels of phosphorylated cJun, but did not influence total cJun expression. However, type I collagen protein and mRNA levels were not affected by the addition of SP600125 to co-cultured cells (figure 3.8 A & B). These results suggest that the JNK signalling pathway is not involved in MDA-MB-231 tumour-mediated down-regulation of type I collagen gene expression in CCD-1068SK fibroblasts.

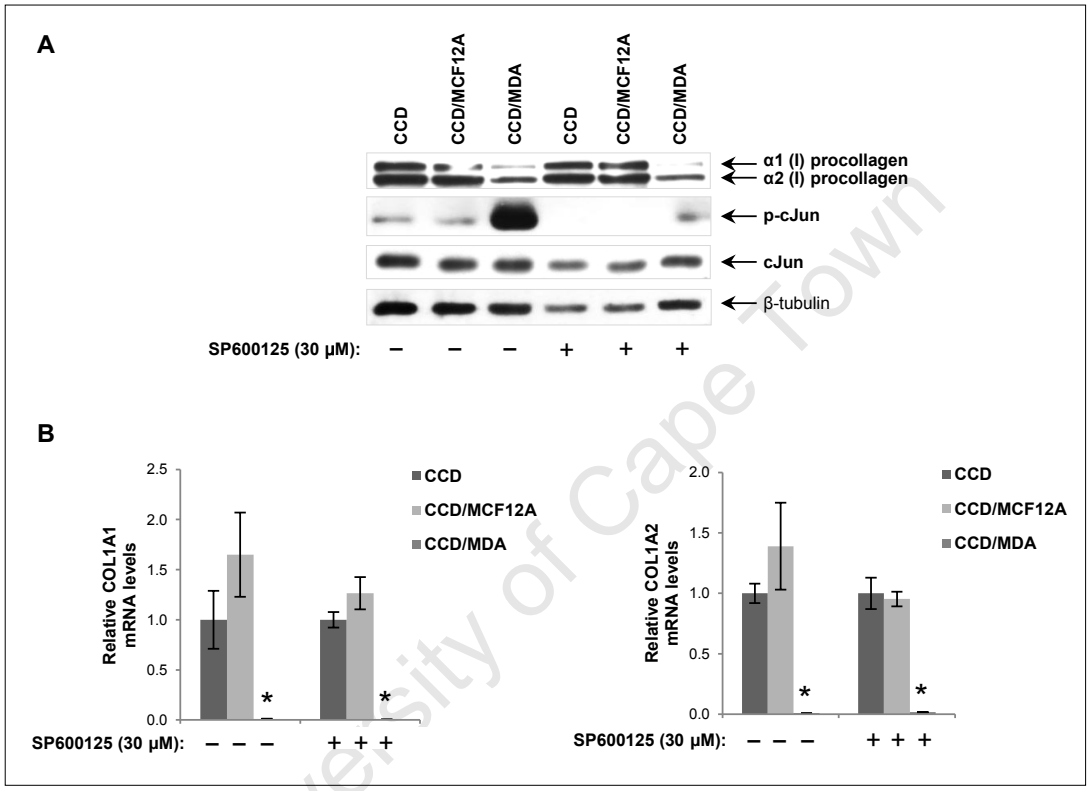

FIGURE 3.8 The effect of JNK pathway inhibition on type I collagen mRNA and protein levels in fibroblast/tumour co-cultures. The JNK inhibitor, SP600125, was incubated with CCD-1068SK/MDA-MB-231 co-cultures for 24 hours before isolating mRNA and protein from cells for further analysis. CCD-1068KS monocultures and co-cultures with MCF12A cells were used as controls. (A) Immunoblotting result shows the effect of SP600125 on type I procollagen, as well as phosphorylated (Ser63/73) and total cJun levels. (B) Real-time PCR results show COL1A1 and COL1A2 mRNA levels in CCD-1068SK fibroblasts co-cultured with tumour cells, in the presence of 30 μM SP600125. The graphs show the mean ± SD from a representative experiment (*$p \leq 0.05$, $n=3$).

3.2.8.2 MEK/ERK signalling in co-cultures.

The MEK/ERK signalling pathway is involved in type I collagen down-regulation in fibroblasts as a result of EGF (Mimura et al. 2006), lysophosphatidic acid (Sato et al. 2004) or C2-ceramide stimulation (Reunanen et al. 2000). Work in our laboratory has also shown that the MEK/ERK pathway is involved in negatively regulating type I collagen

expression in fibroblasts cultured on a fibroblast-derived matrix (Dzobo et al. 2011). However, the exact mechanism whereby ERK regulates type I collagen gene expression is still not well understood.

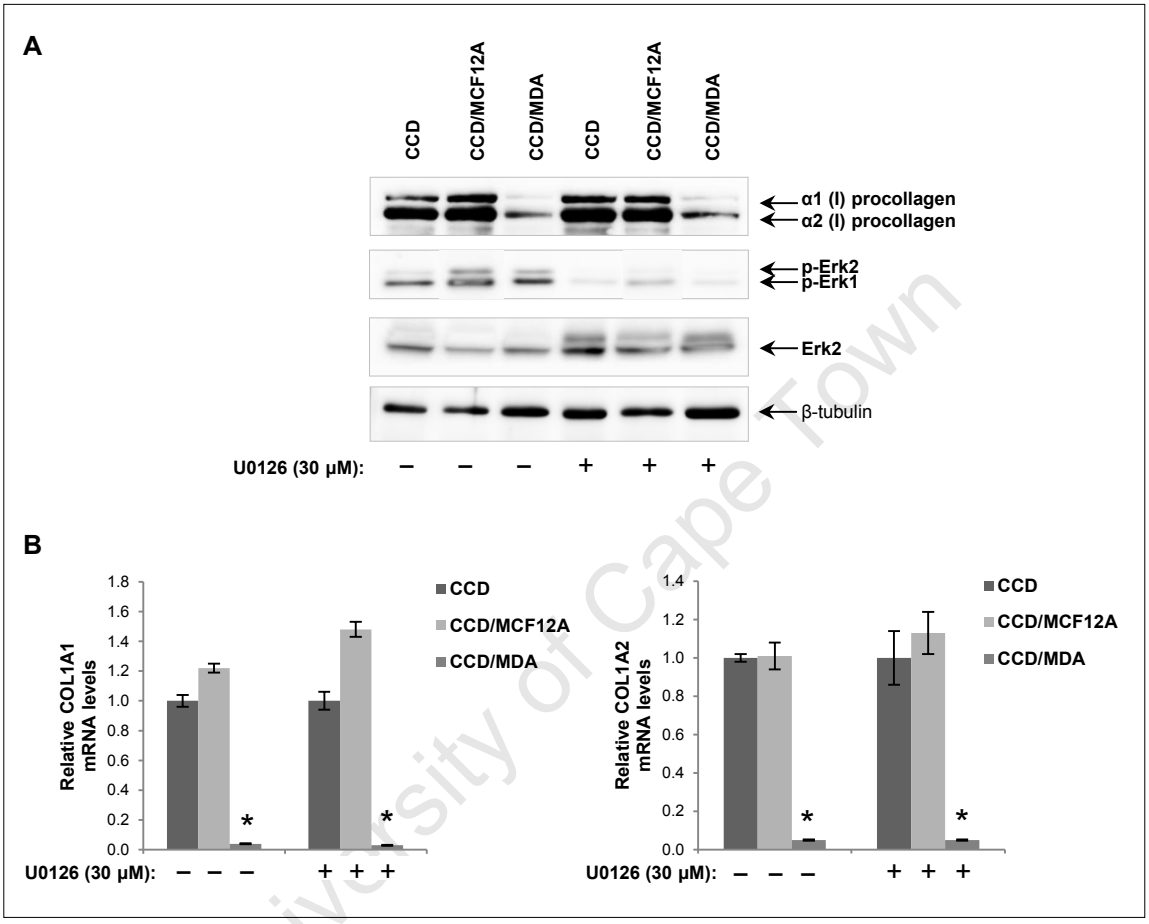

FIGURE 3.9 The effect of MEK/ERK pathway inhibition on fibroblasts/tumour co-culture. CCD-1068SK fibroblast were co-cultured with MDA-MB-231 tumour cells for 24 hours in the presence of the MEK inhibitor, U0126, before isolating RNA and protein for further analysis. CCD-1068SK monocultures and co-cultures with MCF12A cells were used as controls. **(**A) Immunoblotting result showing the effect of U0126 on type I procollagen as well as phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK2 levels. (B) Real-time PCR results show the effect of U0126 on COL1A1 and COL1A2 mRNA levels. The graphs show the mean ± SD from a representative experiment (*$p \leq 0.05$, $n=3$).

To determine the role of the MEK/ERK signalling pathway in MDA-MB-231-tumour mediated type I collagen gene regulation, CCD-1068SK/MDA-MB-231 co-cultured cells were incubated with U0126, which specifically inhibits MEK1 and MEK2 activation (section 6.16). Immunoblotting analysis was performed using a phospho-ERK 1/2 (Thr202/Tyr204) antibody and results showed that levels of activated ERK 1/2 were not

significantly affected by direct co-culturing of CCD-1068SK fibroblasts with MDA-MB-231 tumour cells or MCF12A epithelial cells (figure 3.9 A). Addition of U0126 was also shown to effectively inhibited ERK1/2 phosphorylation in all cell cultures, with no significant effect on total ERK2 levels observed. Endogenous type I procollagen and mRNA levels in co-cultured fibroblasts were not affected by the addition of U0126 to co-cultured cells (figure 3.9 A & B). Therefore, the MEK/ERK signalling pathway does not appear to be involved in tumour-mediated regulation of type I collagen gene expression.

3.2.8.3 p38 MAPK signalling in co-cultures.

The p38 MAPK pathway is often activated in response to physical stress and also plays an important role in cell proliferation, differentiation, apoptosis and inflammation (Zhang et al. 2007a). Previous studies have shown that p38 is involved in the activation of type I collagen gene expression in response to TGFβ stimulation (Abecassis et al. 2004; Hanafusa et al. 1999; Inagaki & Okazaki 2007; Sato et al. 2002). However, type I IFNs are also known to activate p38 signalling (Katsoulidis et al. 2005) and since IFNα and IFNβ gene expression was up-regulated in CCD-1068SK/MDA-MB-231 co-cultures, we investigated the possible role of p38 in negatively regulating type I collagen gene expression in a tumour cell-dependent manner.

CCD-1068SK/MDA-MB-231 co-cultures were incubated with SB203580, which is a pyridinyl imidazole inhibitor that prevents p38 phosphorylation by competing for the ATP-binding site on p38 (specifically p38α and p38β) (section 6.16). Immunoblotting analysis performed on isolated protein showed that phosphorylated p38 (Thr180/Tyr182) as well as total p38 protein levels were the same in CCD-1068SK fibroblasts monocultures and in co-cultures with either MCF12A or MDA-MB-231 tumour cells (figure 3.10 A). Addition of SB203680 to cells resulted in almost complete inhibition of p38 activation, but did not affect total p38 levels. Type I collagen protein levels were, however, not significantly influenced by the addition of SB203580 to CCD-1068SK/MDA-MB-231 co-cultures. Analysis of type I collagen mRNA levels showed that SB203580 addition to fibroblast/tumour co-cultures caused a 2-fold increase in levels of both COL1A1 and COL1A2 when compared to levels in control fibroblast/tumour co-cultures (figure 3.10 B).

However, mRNA levels of COL1A1 and COL1A2 in SB203580-inhibited co-cultured fibroblasts were still significantly down-regulated in comparison to levels in control CCD-1068SK fibroblasts.

These results suggest that p38 may play a part in tumour-mediated negative regulation of type I collagen levels in neighbouring fibroblasts, but that other signalling pathways and/or transcription factors are also involved.

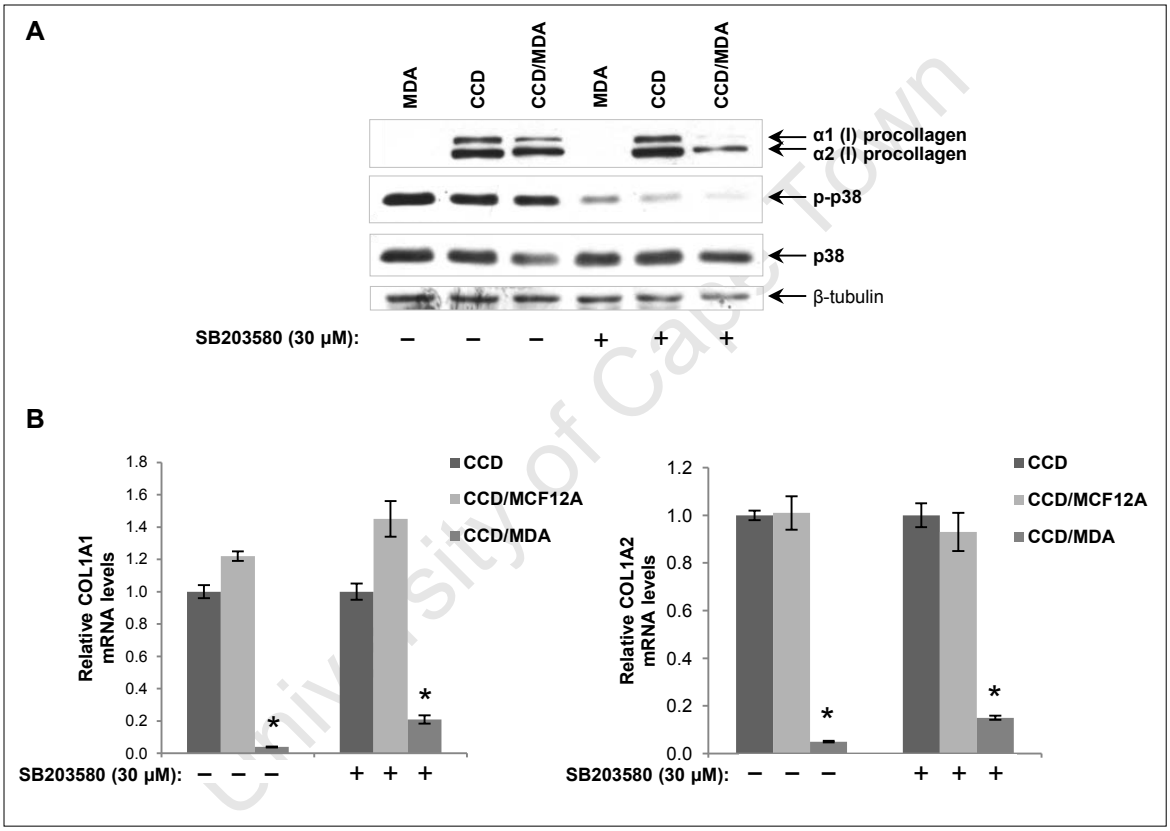

FIGURE 3.10 The effect of p38 MAPK pathway inhibition on type I collagen mRNA and protein levels in fibroblast/tumour co-cultures. CCD-1068SK fibroblasts were co-cultured with MDA-MB-231 tumour cells for 24 hours in the presence of SB203580 **(+)**. CCD-1068SK monocultures and co-cultures with MCF12A cells were used as controls **(-)**. (A) Immunoblotting results show the effect of SB203580 on type I procollagen levels as well as phosphorylated p38 MAPK (Thr180/Tyr182) and total p38 MAPK levels. (B) Real-time PCR results show the effect of SB203580 on COL1A1 and COL1A2 mRNA levels in CCD-1068SK/MDA-MB-231 co-cultures. The graphs show the mean ± SD from representative experiments and significance was calculated relative to SD values for CCD-1068SK monocultures and CCD-1068SK/MDA-MB-231 co-cultures (*$p \leq 0.05$, $n=3$).

3.3 DISCUSSION

In this study we show that close contact between CCD-1068SK fibroblasts and MDA-MB-231 tumour cells results in increased type I IFN and Stat1 gene expression as well as Stat1 protein activation in both fibroblasts and tumour cells. The increased expression and activation of Stat1 observed in fibroblasts was shown to only occur as a result of factors secreted during direct fibroblast/tumour co-culture, which suggests that type I IFNs could be involved in mediating this response. However, inhibition studies and indirect co-culture experiments showed that the increased Stat1 activity observed in tumour/fibroblast co-cultures did not affect type I collagen gene expression, which suggests that the JAK/Stat signalling pathway is not involved in mediating the negative regulatory effect of tumour cells on type I collagen gene expression.

Based on the results obtained, we propose a model for the inflammatory response occurring during fibroblast/tumour co-cultures (figure 3.11). Firstly, a tumour-produced factor causes increased IFNα and IFNβ gene expression in fibroblasts that are in close contact with tumour cells. These type I IFNs are then secreted into the medium and, since these cytokines can signal in both an autocrine and paracrine fashion (Stetson and Medzhitov 2006), they bind to type I IFN receptors on both the fibroblasts and tumour cell surfaces and activate the JAK/Stat signalling pathway. As a result, Stat1 gene expression and activity is increased, which leads to a further increase in type I IFN gene expression in both cell types, particularly of IFNβ. Previous microarray studies performed using MDA-MB-231/fibroblast co-cultured cells have shown that a set of interferon response genes, including Stat1, is up-regulated during direct co-culture (Buess et al. 2007). Immunohistochemical studies also showed that Stat1 was strongly induced in a subset of breast tumour cells, specifically at the tumour-stroma border, and this increased Stat1 expression was inversely correlated with poor patient outcome. Our findings support those of Buess et al. (2007) and suggest that our co-culture system reflects *in vivo* conditions.

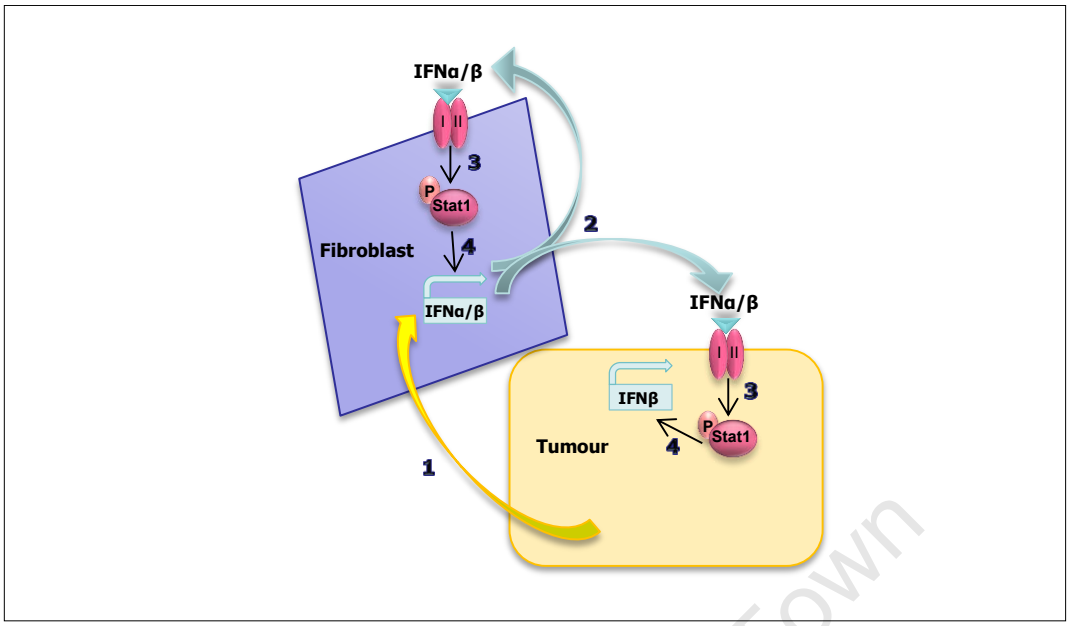

FIGURE 3.11 A proposed model showing the interferon response occurring when fibroblasts are directly co-cultured with tumour cells. When MDA-MB-231 tumour cells are directly co-cultured with CCD-1068SK fibroblasts, the tumour cells produce an unknown factor that results in an increase in IFNα and IFNβ expression in neighbouring fibroblasts (1). These interferons are secreted by the fibroblasts into the medium (2) where they bind to type I IFN receptors on both the fibroblast and tumour cell surfaces. This binding activates the JAK/Stat signalling pathway in both cell types (3), with a resulting increase in Stat1 gene expression and protein phosphorylation. JAK/Stat signalling activation causes a further increase in IFNβ expression in tumour cells and in both IFNα and IFNβ gene expression in fibroblasts (4).

Increased production of type I IFNs and activation of the interferon response usually occurs as part of the innate immune response to intracellular pathogens. This response is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR) such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) on the cell surface (Honda et al. 2006; Takaoka & Yanai 2006). PRRs can also be activated in sterile conditions in response to damage-associated molecular patterns (DAMPs) generated by injured or damaged cells (Matzinger 1994; Matzinger 2007). Since DAMP production by tumour cells has also been linked to increased inflammation in the tumour environment (Srikrishna & Freeze 2009) it is possible that DAMPs produced by MDA-MB-231 tumour cells may be detected by PRRs on the fibroblast cell surface, initiating an inflammatory response. However, further studies would need to be performed to determine the exact nature of this tumour-produced factor.

The increase in type I IFN production by fibroblasts in close contact with tumour cells could result in the recruitment of other immune cells to the tumour site. In fact, fibroblasts

are regarded as sentinel cells in chronic inflammation because they aid in the migration of leukocytes to the wound site (Smith et al. 1997). Type I IFNs are usually regarded as a tumour suppressor and have been used for the treatment of tumours (Takaoka & Yanai 2006), however recruitment of immune cells to the tumour site does not always result in tumour eradication and the results may be context dependent. Chronic activation of certain types of innate immune cells at the tumour site, such as tumour-associated macrophages (TAMs), could result in a proinflammatory tumour environment and facilitate further tumour invasion and metastasis (de Visser et al. 2006). This type of inflammation has previously been observed in breast tumours in which no pre-existing inflammation was found (Mantovani et al. 2008).

The increased interferon gene expression observed in CCD-1068SK/MDA-MB-231 co-cultures was accompanied by an increase in Stat1 phosphorylation in both cell types. Since increased Stat1 activation did not lead to any changes in type I collagen gene expression in fibroblasts exposed to secreted factors from tumour/fibroblast co-cultures, the JAK/Stat signalling pathway does not appear to be involved in tumour-mediated type I collagen regulation. These results also confirm that negative regulation of type I collagen gene expression is not a result of a secondary response induced by factors secreted during fibroblasts/tumour direct co-cultures, but that this negative regulatory effect occurs as a direct result of close contact between tumour cells and fibroblasts.

Analysis of cytokines secreted in fibroblast/tumour direct co-culture medium showed that previously characterised negative regulators of type I collagen gene expression such as TNFα, IFNγ and IL1 are also not involved in tumour-mediated type I collagen suppression in fibroblasts. Further examination of MAPK signalling pathways in fibroblast/tumour co-cultures revealed that JNK and MEK signalling did not play a role in tumour-mediated type I collagen regulation, but that the p38 MAPK signalling pathway may be partially involved. Since the inhibitors used in these experiments were added to co-cultured cells, they would inhibit the specific MAPK in both fibroblasts and tumour cells. Therefore, further investigation of the role of p38 MAPK signalling would require selective inhibition of the p38 protein in either fibroblasts or tumour cells.

Our investigations have revealed that a number of cytokines known to be negative regulators of type I collagen gene expression are not involved in MDA-MB-231 tumour-mediated regulation in neighbouring fibroblasts. Since close contact between fibroblasts and tumour cells is required for negative regulation of type I collagen synthesis, it is possible that cell-surface receptors and cell matrix proteins may be involved in regulation. Further investigation of these factors may lead to a better understanding of type I collagen regulation by tumour cells.

CHAPTER FOUR

THE EFFECT OF BREAST TUMOUR CELLS ON THE EXPRESSION OF ADHESION AND ECM COMPONENTS IN CO-CULTURED FIBROBLASTS.

4.1 INTRODUCTION

Invading tumour cells could communicate with surrounding fibroblasts in a number of different ways, with the proximity to these cells playing a role in the types of signalling that could take place. Apart from paracrine signalling to neighbouring fibroblasts via secreted factors, tumour cells in closer proximity to fibroblasts could also communicate via cell adhesion molecules, which could lead to changes in the expression of extracellular matrix genes such as type I collagen.

Adhesion molecules such as integrins and cadherins are known mediators of cell-matrix and cell-cell signalling. Integrins may bind to ECM proteins and in so doing assist in cell adhesion and in communicating signals from the surrounding ECM to the cell. Integrins α1β1, α2β1, α10β1 and α11β1 are the most well-known receptors for collagens and can be differentiated from other integrin subunits by the extra subdomain (Iα) in their α-subunit, which binds to collagen in the ECM (Heino 2000). Integrins are also able to associate with other cell adhesion molecules on adjacent cells, including immunoglobulin-type receptors ICAMs (intercellular adhesion molecules) and VCAMs (vascular cell adhesion molecules), as well as members of the ADAM (a disintegrin and metalloprotease) family (Bernstein 1998; Danen 2005; Heino 2000; Oberyszyn et al. 1998). Another group of cell surface receptors are the cadherins, which are mainly involved in cell-cell adhesion. N-cadherin has specifically been detected in a number of tumours and has been shown to be involved in melanoma cells adhesion to dermal fibroblasts and vascular endothelial cells (Li et al. 2001). Although changes in the gene expression of cell adhesion proteins have been associated with tumour initiation, growth and metastasis (Danen 2005), the role of these proteins in tumour cell communication with neighbouring fibroblasts and how this could influence ECM synthesis by fibroblasts is still not completely understood.

Another group of proteins that play a role in modulating cell-ECM interactions and ECM synthesis are the extracellular-matrix associated or "matricellular" proteins (Bornstein & Sage 2002), which include TSP-1 (thrombospondin-1), SPARC (secreted protein, acidic and rich in cysteine), tenascin-C, osteopontin and members of the CCN family. Matricellular proteins are secreted into the ECM and, although they do not play a direct role in maintaining structural integrity (Bornstein 2009), they are involved in regulating the cell's response to other environmental factors and can interact with cell surface receptors, ECM components, growth factors, cytokines and proteases (Bornstein & Sage 2002; Chen & Lau 2009). Connective tissue growth factor (CTGF/CCN2) is a matricellular protein that is involved in matrix remodelling and is often implicated in fibrosis (Shi-wen et al. 2000). CCN2 can signal through integrins and heparin-sulfate proteoglycans (HSPG) (Leask et al. 2009), although the exact signalling mechanisms may vary according to cell type and conditions in the cellular environment (Shi-wen et al. 2008). CCN2 gene expression can be induced by TGF-β, and CCN2 also acts as a co-mediator of TGFβ-stimulated type I collagen gene expression (Igarashi et al. 1993; Leask et al. 2003; Shi-wen et al. 2000).

TGFβ binding to its receptors activates the Smad signalling pathway which positively regulates both CCN2 and type I collagen gene expression in fibroblasts (Hishikawa et al. 2007; Leask et al. 2003; Ramirez et al. 2006). The Smad3/4 complex binds to the CCN2 promoter, while the Smad2/3/4 complex associates with p300 to positively regulate COL1A2 promoter activity (figure 4.1) (Holmes et al. 2001; Inagaki & Okazaki 2007; Zhang et al. 2000). COL1A1 is also known to be a Smad3/4 gene target and a Smad binding element has been identified in the COL1A1 promoter (Verrecchia et al. 2001a; Lakos et al. 2004). An intact Smad signalling pathway is also reported to be essential for CCN2-mediated regulation of type I collagen gene expression in skin fibroblasts (Quan et al. 2009). Smad7 is a well-known negative regulator of the Smad signalling pathway that is able to interfere with R-Smad/TGFβ receptor interactions and Smad-Smad interactions, as well as induce TGFβRI degradation (Hayashi et al., 1997; Heldin et al. 2009; Leask & Abraham 2004; Nakao et al. 1997; Shi et al. 2004). Overexpression of Smad7 has

previously been found to decrease COL1A1, COL1A2 and CCN2 gene expression (van Beek et al. 2006; Chen et al. 1999; Holmes et al. 2001; Quan et al. 2009).

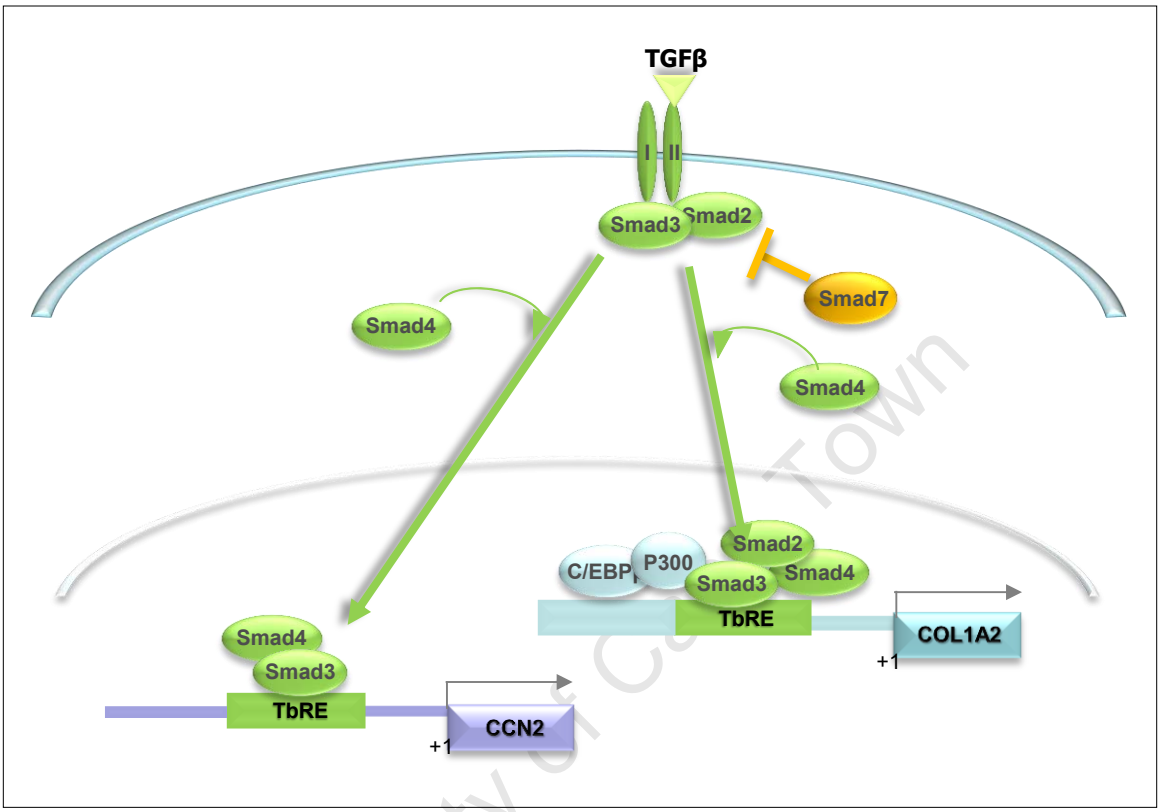

Figure 4.1. TGFβ-mediated regulation of CCN2 and COL1A2 gene expression. Binding of TGFβ to the TGFβ-receptor complex results in the phosphorylation of Smad2 and Smad3, which form a complex with Smad4 and translocate to the nucleus. The Smad2/3/4 complex associates with p300 and binds to the TbRE (TGFβ response element) of the COL1A2 promoter, resulting in increased transcription of COL1A2. CCN2 promoter activity is also increased when the Smad3/4 complex binds to the CCN2 promoter. Smad7 acts as a negative feedback inhibitor of the Smad signalling pathway.

The aim of this chapter was to investigate the possible role of adhesion molecules and ECM components in MDA-MB-231 tumour-mediated suppression of type I collagen gene expression in CCD-1068SK fibroblasts. Since there is not much information available on how fibroblast/tumour co-culture affects ECM or adhesion molecules, a microarray was used to specifically examine the expression of ECM and cellular adhesion molecule genes in CCD-1068SK fibroblast after direct co-culture with MDA-MB-231 cells. Further studies involved determining the role of CCN2 and Smad7 in mediating the negative regulatory effect of MDA-MB-231 tumour cells on type I collagen gene expression in CCD-1068SK fibroblasts.

4.2 RESULTS

4.2.1 ECM and adhesion molecule gene expression in fibroblasts after tumour cell co-culture.

Our earlier results show that MDA-MB-231 tumour cells require close contact with normal fibroblasts to exert a negative regulatory effect on type I collagen gene expression. It is therefore possible that cell surface molecules, such as adhesion proteins, could be involved in triggering the tumour-induced inhibitory effect on type I collagen synthesis. The effect of direct co-culture with MDA-MB-231 tumour cells on cell adhesion molecule and ECM molecule gene expression in CCD-1068SK fibroblasts was therefore further investigated.

CCD-1068SK fibroblasts were directly co-cultured with MDA-MB-231 tumour cells and separated by FACS. RNA was isolated from CCD-1068SK fibroblasts and used to profile the expression of 113 genes (table 4.1) that form part of the Oligo GEArray® Human Extracellular Matrix and Adhesion Molecules microarray (SABiosciences), as described in Materials and Methods (section 6.17). The expression images obtained were uploaded onto the web-based GEArray Expression Analysis Suite for data analysis (figure 4.2).

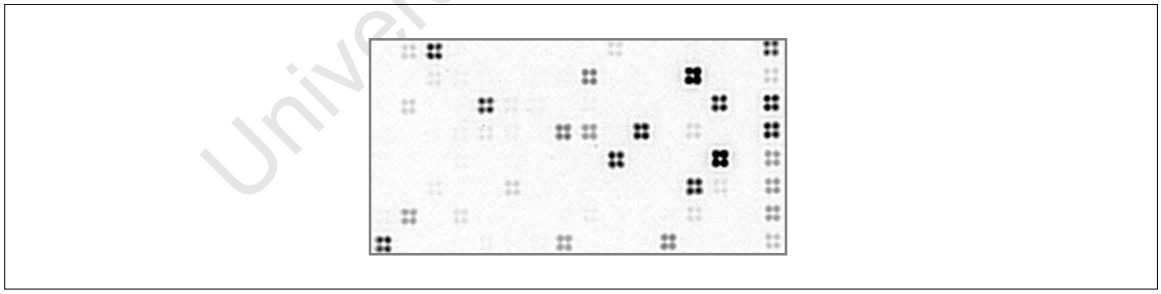

FIGURE 4.2 A representative chemiluminescent image of an OligoGE nylon membrane array. Sample oligonucleotide probes were hybridised to OligoGE membrane arrays and detected with CDP-Star. Each cluster of 4 spots represents one gene, with a total of 128 genes represented on the array.

Table 4.1. Genes included on the Extracellular Matrix and Adhesion Molecules Oligo GEarray.
Abbreviations: HAS, hyaluronan synthase; ICAM, intercellular adhesion molecule; NCAM, neural cell adhesion molecule; PECAM, platelet endothelial cell adhesion; VECAM, vascular endothelial cell adhesion molecule; SELE, E-selectin; SELL, L-selectin; SELP, P-selectin; ITGA, integrin α; ITGB, integrin β; MMP, matrix metalloproteinase; SGCE, episilon-sarcoglycan; SPG7, paraplegin; COL, collagen; CTNN, catenin; ADAMTS, A disintegrin and metalloproteinase with thrombospondin motifs; SPP, stromal processing peptidase; THBS, thrombospondin; CNTN, contactin; VCAN, versican; FN, fibronectin; KAL1, anosmin-1; LAMA, laminin α; LAMB, laminin β; CLEC, Tetranectin; VTN, vitronectin; TIMP, tissue inhibitors of metalloproteinases; ECM, extracellular matrix protein.

Transmembrane Molecules	CD44, CDH1, SGCE HAS1, ICAM1, NCAM1, PECAM1, VCAM1, SELE, SELL, SELP ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, MMP14, MMP15, MMP16, SPG7
Cell-Cell Adhesion	CD44, CDH1, COL11A1, COL14A1, COL6A2, CTNND1, ICAM1, VCAM1 ITGA8
Cell-Matrix Adhesion	ADAMTS13, CD44, SGCE ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, SPP1, THBS3.
Other Adhesion Molecules	CNTN1, VCAN, CLEC3B CCN2, THBS1, THBS2, TNC CTNNA1, CTNNB1, CTNND2,
Collagens & ECM Structural Constituents	COL11A1, COL12A1, COL14A1, COL15A1, COL16A1, COL1A1, COL4A2, COL5A1, COL6A1, COL6A2, COL7A1, COL8A1, FN1, KAL1.
ECM Proteases	ADAMTS1, ADAMTS13, ADAMTS8, MMP1, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP2, MMP3, MMP7, MMP8, MMP9, SPG7, TIMP1
ECM Protease Inhibitors	COL7A1, KAL1, THBS1, TIMP1, TIMP2, TIMP3
Basement Membrane Constituents	COL4A2, COL7A1, LAMA1, LAMA2, LAMA3, LAMB1, LAMB3, LAMC1, SPARC.
Other ECM Molecules	VCAN, CLEC3B CCN2, THBS2, THBS3, TNC ECM1, VTN HAS1, SPP1, TGFBI

Expression analysis revealed that very few of the genes on the array were differentially regulated. MMP-1 gene expression was however shown to be up-regulated in co-cultured CCD-1068SK fibroblasts, while the expression of a number of collagen genes was down-regulated in response to tumour co-culture (table 4.2). Although COL1A2 was not included on the array, the down-regulation of COL1A1 observed in co-cultured CCD-1068SK fibroblasts confirmed our earlier results. Microarray results also showed that CCN2 was substantially decreased in fibroblasts co-cultured with tumour cells. The microarray findings were independently confirmed by means of real-time PCR analysis. MMP-1 gene expression was significantly up-regulated in co-cultured fibroblasts (figure 4.3 A), while CCN2 mRNA levels were decreased more than 5-fold in fibroblasts in close contact with tumour cells (figure 4.3 B).

Table 4.2 Differential gene expression in CCD-1068SK fibroblasts after direct co-culture with MDA-MB-231 tumour cells.

	CCD(MDA)/CCD
MMP-1	1.26
COL1A1	0.50
COL18A1	0.40
COL6A3	0.36
COL12A1	0.29
COL4A2	0.18
COL8A1	0.14
COL5A3	0.05
CCN2	0.0001

These results suggest that tumour cells affect fibroblast genes involved in ECM remodelling. Since the CCN2 protein has previously been shown to play a role in type I collagen gene expression, CCN2 may be involved in mediating the negative regulatory effect of MDA-MB-231 tumour cells on type I collagen gene expression in fibroblasts.

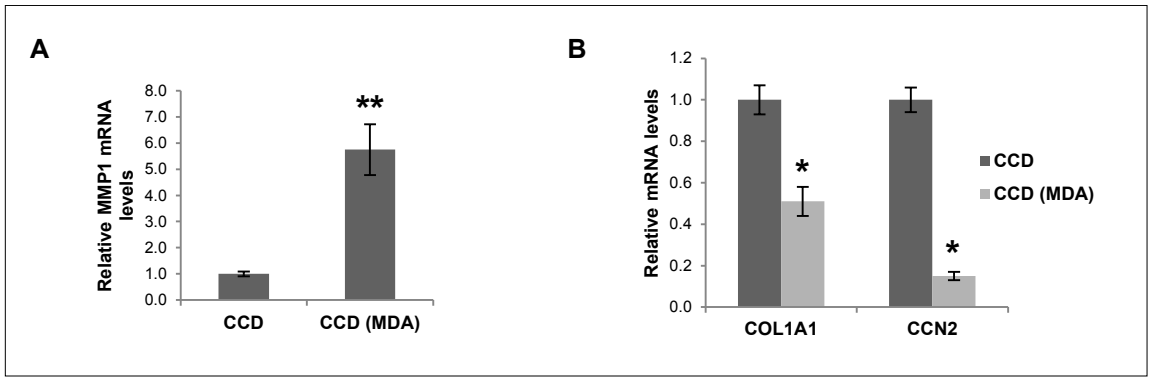

Figure 4.3 MMP1, COL1A1 and CCN2 mRNA levels in fibroblasts after direct co-culture with tumour cells. CCD-1068SK fibroblasts were directly co-cultured with MDA-MB-231 tumour cells for 48 hours, followed by separation of cell types again by FACS. Real-time PCR analysis was carried out to determine relative MMP-1 (A), COL1A1 and CCN2 (B) mRNA levels in co-cultured CCD-1068SK fibroblasts. The graphs show the mean ± SD from representative experiments (* $p \leq 0.05$, ** $p \leq 0.01$; $n=3$). Abbreviations: CCD, CCD-1068SK; CCD(MDA), CCD-1068SK fibroblasts after co-culture and FACS separation from MDA-MB-231 cells.

4.2.2 The effect of CCN2 on type I collagen gene expression.

CCN2 is a matricellular protein that has been reported to be a co-mediator of TGF-β's ability to promote ECM synthesis, including that of type I collagen (Blom et al. 2002). CCN2 is overexpressed in a number of fibrotic disorders such as scleroderma and has been well studied in these diseases (Abraham 2008; Khoo et al. 2006; Leask et al. 2009; Ponticos et al. 2009; Uchio et al. 2004). Previous studies suggest that, in fibroblasts, TGFβ stimulates CCN2 gene expression, which facilitates in increasing type I collagen gene expression in response to TGFβ (Khoo et al. 2006; Ponticos et al. 2009; Quan et al. 2009). Since our microarray and RT-PCR results showed that both type I collagen and CCN2 mRNA levels were significantly decreased in CCD-1068SK fibroblasts directly co-cultured with MDA-MB-231 tumour cells, we investigated a possible role for CCN2 in tumour-mediated type I collagen regulation.

4.2.2.1 CCN2 gene expression in tumour/fibroblast co-cultures.

CCN2 is a 36-38 kDa protein, depending on its degree of glycosylation, which is either secreted through the Golgi apparatus into the stroma or remains attached to the cell surface and ECM (Blom et al. 2002). CCN2 has been observed as homodimers (~70-80 kDa) or as cleavage products (16-20 kDa or 10-12 kDa), which are still biologically active (Ball et al. 2003; Khoo et al. 2006; Rickelt et al. 2008; Holbourn et al. 2008; Tikellis et al. 2004;

Wahab et al. 2001) and much of the variation found in CCN2 appears to be cell type specific (Blom et al. 2002). We therefore wanted to determine how tumour/fibroblast co-culture was affecting CCN2 production in both cell types, as well as secretion of CCN2 into the medium.

To determine CCN2 protein levels in CCD-1068SK fibroblasts and MDA-MB-231 tumour cells after direct co-culture, immunoblotting analysis was performed on total endogenous protein isolated from both cell types after FACS separation. A human anti-CCN2 antibody against the region mapping within amino acids 150-200 was used for the analysis. This region contains the hinge joining the N- and C-terminal domains as well as amino acids from both domains. Results of immunoblotting identified the 36-38 kDa CCN2 protein as present in both CCD-1068SK fibroblasts and MDA-MB-231 tumour cells, with the double band representing differences in CCN2 glycosylation (figure 4.4 A & B). Direct co-culture of CCD-1068SK fibroblasts with tumour cells resulted in a decrease in CCN2 levels when compared to levels in control fibroblasts (figure 4.4 A). However, CCN2 levels in co-cultured MDA-MB-231 tumour cells were similar to those observed in tumour monocultures (figure 4.4 B).

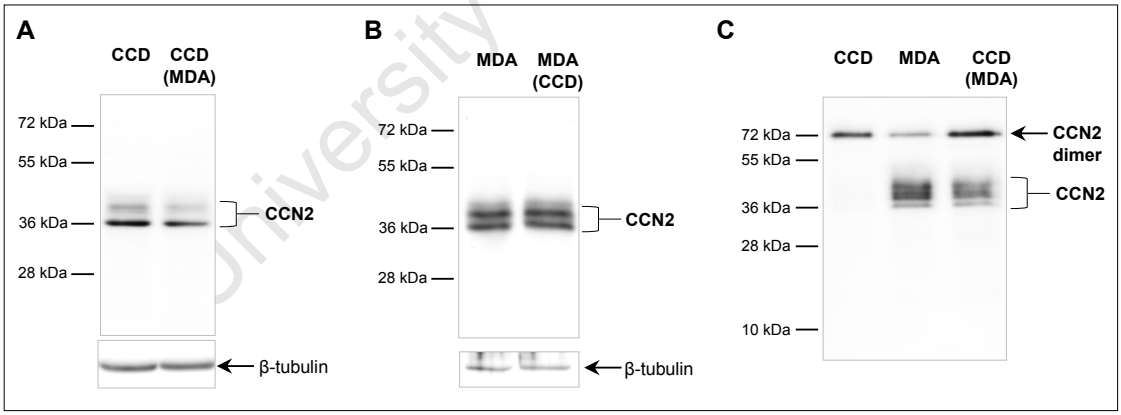

FIGURE 4.4 CCN2 protein levels in fibroblast/tumour direct co-cultures. CCD-1068SK fibroblasts were directly co-cultured with MDA-MB-231 tumour cells for 48 hours and then separated by FACS. Equivalent amounts of total cell lysate were used for immunoblotting analysis with the CCN2 antibody as described in Materials and Methods (section 6.11). CCN2 was expressed as a 36-38 kDa protein in CCD-1068SK fibroblasts (A) and MDA-MB-231 tumour cells (B). CCN2 protein was isolated from CCD-1068SK/MDA-MB-231 co-culture and monoculture cell medium and equal protein was used for immunoblotting analysis (C). CCN2 was detected at both 36-38 kDa and at ~72 kDa. Abbreviations: CCD, CCD-1068SK; MDA, MDA-MB-231; CCD(MDA), CCD-1068SK fibroblasts after co-culture and FACS separation from MDA-MB-231 cells; MDA(CCD), MDA-MB-231 tumour cells after co-culture and FACS separation from CCD-1068SK fibroblasts.

Since CCN2 is a matricellular protein that is thought to perform most of its functions in the ECM, immunoblotting analysis was performed to determine the levels of CCN2 present in the medium of CCD-1068SK/MDA-MB-231 co-cultures and monocultures of each cell type after 48 hours incubation. Results showed that CCN2 found in control CCD-1068SK medium occurred mainly as a ~72 kDa fragments, presumed to be CCN2 dimers, with no 36-38 kDa form detected (figure 4.4 C). In control MDA-MB-231 cell medium, the 72 kDa band was present to a lesser degree, compared to the monomeric form of CCN2. CCD-1068SK/MDA-MB-231 co-culture cell medium showed all forms observed in both cell type monocultures.

These results suggest that CCN2 is expressed as a 36-38 kDa protein in both fibroblast and tumour cell monocultures, but that endogenous CCN2 levels are lower in CCD-1068SK fibroblasts co-cultured with MDA-MB-231 tumour cells, as observed at an mRNA level. Since decreased CCN2 levels may cause decreased type I collagen levels under co-culture conditions, we next investigated the effect of inhibiting fibroblast CCN2 expression on type I collagen gene expression.

4.2.2.2 The effect of CCN2 inhibition on type I collagen gene expression.

To determine whether decreased CCN2 gene expression influences type I collagen gene expression in CCD-1068SK fibroblasts, these cells were transfected with CCN2 siRNA and incubated for 48 hours before harvesting protein.

Immunoblotting results showed that CCN2 protein levels were decreased by up to 90% in CCD-1068SK fibroblasts transfected with 40 nm CCN2 siRNA (figure 4.5 A & B). The effect of silencing CCN2 on type I collagen gene expression only became apparent at 40 nm CCN2 siRNA and above, which resulted in a two-fold decrease in type I collagen protein levels (figure 4.5 A & C). CCN2 mRNA levels were also significantly decreased in fibroblasts transfected with 40 nm CCN2 siRNA (figure 4.5 D). As observed at a protein level, CCN2 inhibition resulted in a 2-fold decrease in COL1A1 mRNA levels, although COL1A2 mRNA levels were only slightly lower than those observed in control fibroblasts.

Since silencing CCN2 expression in CCD-1068SK fibroblasts resulted in a decrease in type I collagen mRNA and protein levels, it is possible that MDA-MB-231 tumour-mediated negative regulation of CCN2 gene expression in fibroblasts could, in turn, result in the decreased type I collagen gene expression observed in co-cultured CCD-1068SK fibroblasts.

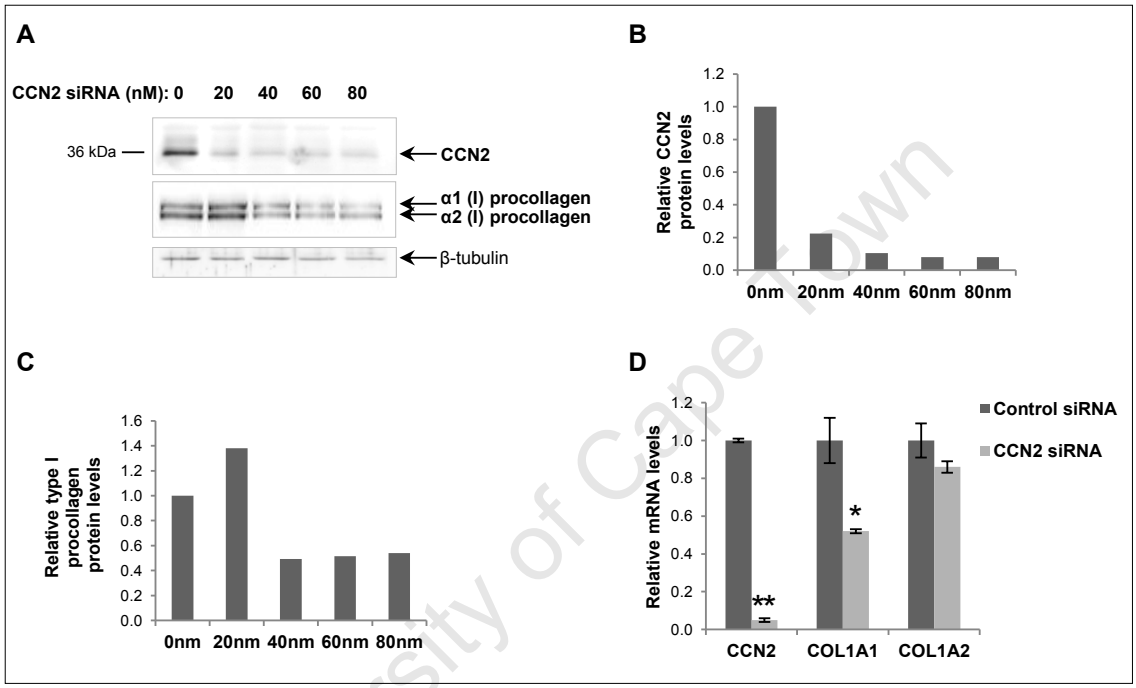

FIGURE 4.5 Silencing CCN2 expression in CCD-1068SK fibroblasts. CCD-1068SK fibroblasts were transfected with varying concentrations of CCN2 siRNA and incubated for 48 hours. (A) Immunoblotting analysis of endogenous CCN2 and type I procollagen protein levels in CCD-1068SK fibroblasts transfected with CCN2 siRNA. (B) Quantitation of relative CCN2 levels. (C) Quantitation of relative type I procollagen levels. (D) Real-time PCR analysis of CCN2, COL1A1 and COL1A2 mRNA levels in CCD-1068SK fibroblasts transfected with CCN2 siRNA. The graph shows the mean ± SD from a representative experiment (*$p \leq 0.05$, ** $p \leq 0.01$; $n=3$).

4.2.2.3 The effect of Smad7 on CCN2 and type I collagen gene expression.

The Smad signalling pathway plays an important role in the positive regulation of both CCN2 and type I collagen gene transcription, with TGFβ being the most well-known activator of this signalling pathway. The important relationship between TGFβ signalling and CCN2 and type I collagen gene expression was also highlighted by a recent study showing that the expression of all three of these proteins was significantly down-regulated

in aged human skin samples (≥ 80 years) (Quan et al. 2009). Smad7 is known to act as a negative regulator of the Smad signalling pathway, and an increase in Smad7 gene expression may therefore result in suppression of both CCN2 and type I collagen gene expression. As we observed a decrease in both CCN2 and type I collagen gene expression in co-cultured fibroblasts, the potential role of Smad7 in tumour-mediated regulation of these genes was further investigated.

4.2.2.3.1 The effect of tumour cells on Smad7 gene expression in co-cultured fibroblasts.

To determine if tumour cells caused any change in Smad7 mRNA and/or protein levels in co-cultured fibroblasts, MDA-MB-231/CCD-1068SK co-cultures were incubated for 48 hours and separated by means of FACS.

Real-time PCR analysis revealed that tumour cell co-culture caused a significant increase in Smad7 mRNA levels in CCD-1068SK fibroblasts (figure 4.7 A). Smad7 protein levels were also increased in co-cultured fibroblasts, albeit not to the degree observed at an mRNA level (figure 4.7 B & C).

These results show that direct co-culture with MDA-MB-231 tumour cells results in an increase in both the mRNA and protein levels of Smad7 in CCD-1068SK fibroblasts. We speculate that Smad7 may therefore play a role in tumour-mediated negative regulation of both type I collagen and CCN2 synthesis by preventing positive Smad signalling in fibroblasts.

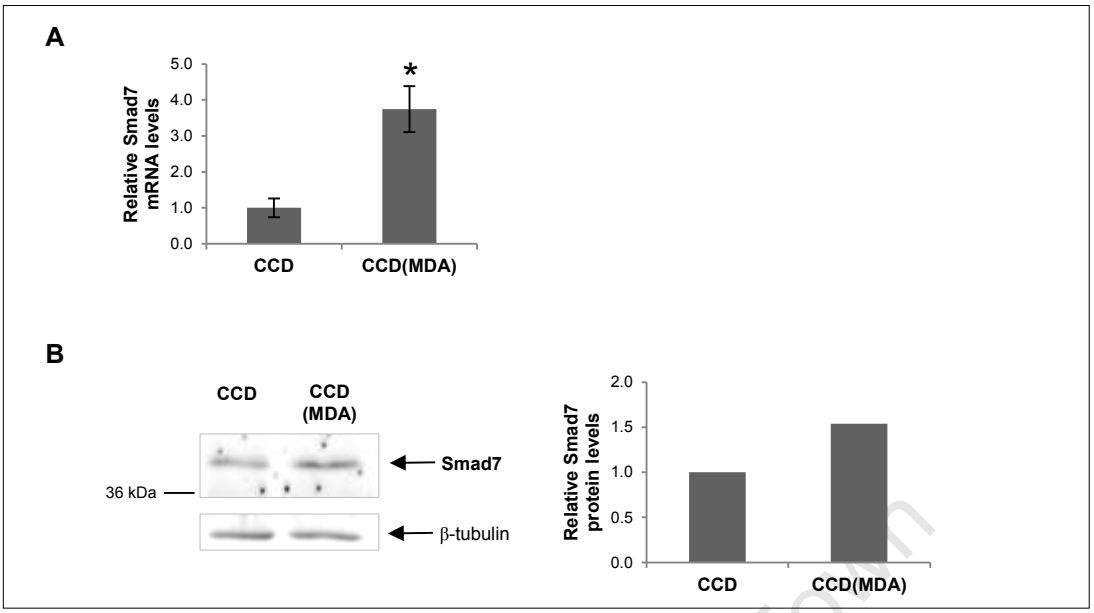

FIGURE 4.7 Smad7 mRNA and protein levels in fibroblasts after direct co-culture with tumour cells. Fluorescently labeled CCD-1068SK fibroblasts were directly co-cultures with MDA-MB-231 tumour cells for 48 hours, followed by FACS separation. RNA and protein was extracted from separated CCD-1068SK fibroblasts for further analysis. (A) Relative Smad7 mRNA levels in co-cultured CCD-1068SK fibroblasts were quantified by means of real-time PCR. The graph shows the mean ± SD from a representative experiment (*$p \leq 0.05$, $n=3$). (* $p \leq 0.05$). (B) Immunoblotting results show Smad7 levels in co-cultured CCD-1068SK fibroblasts. Relative Smad7 levels were also quantified by means of densitometry. Abbreviations: CCD, CCD-1068SK; MDA, MDA-MB-231; CCD(MDA), CCD-1068SK fibroblasts directly co-cultured with MDA-MB-231 cells and separated again by means of FACS.

4.2.2.3.2 Type I collagen and CCN2 gene expression in Smad7 inhibited fibroblasts.

Since Smad7 gene expression was up-regulated in fibroblasts after co-culture with tumour cells, we wanted to determine whether preventing Smad7 gene expression would reverse the negative regulatory effect of the tumour cells on CCN2 and type I collagen gene expression in CCD-1068SK fibroblasts.

Smad7 gene expression was silenced in CCD-1068SK fibroblasts by transfecting cells with Smad7 siRNA, with real-time PCR and immunoblotting results confirming that both Smad7 mRNA and protein levels were decreased as a result (figure 4.8 A & B). Smad7 inhibition led a significant increase in CCN2 mRNA levels and levels of the 72 kDa CCN2 dimer band were substantially increased in Smad7 inhibited cells (figure 4.8 C & D). However, levels of 36-38 kDa CCN2 remained largely unchanged compared to levels in control fibroblasts. Smad7 inhibition also caused an increase in COL1A1 mRNA levels,

although no change in COL1A2 mRNA levels was observed (figure 4.8 E). On the other hand, both α1(I) and α2(I) procollagen levels was increased in Smad7-inhibited fibroblasts (figure 4.8 F).

To determine the involvement of Smad7 in mediating the effects of tumour cells on CCN2 and type I collagen gene expression in co-cultured fibroblasts, Smad7-inhibited CCD-1068SK fibroblasts were co-cultured with MDA-MB-231 cells. As expected, Smad7 mRNA levels were significantly decreased in Smad7-inhibited co-cultures when compared to control co-cultures (figure 4.9 A). Interestingly, real-time PCR analysis revealed that Smad7 inhibition resulted in a significant increase in CCN2 mRNA levels in fibroblast/tumour co-cultures compared to control samples (figure 4.9 B), suggesting that Smad7 was able to revert tumour-mediated repression of CCN2 gene expression. However, neither COL1A1 nor COL1A2 mRNA levels were affected by Smad7 inhibition (figure 4.9 C) and immunoblotting analysis similarly showed that type I procollagen levels remained unchanged when Smad7 expression was decreased (figure 4.9 D & E).

Results of these experiments suggest that Smad7 is involved in negatively regulating basal CCN2 and type I collagen gene expression in CCD-1068SK fibroblasts, although the effect on type I collagen protein levels was more prominent than that observed at an mRNA level. Smad7 also appears to play a role in mediating the negative regulatory effects of tumour cells on CCN2 gene expression in co-cultured CCD-1068SK fibroblasts. However, our results do not provide conclusive evidence that Smad7 is involved in tumour-mediated negative regulation of type I collagen gene expression.

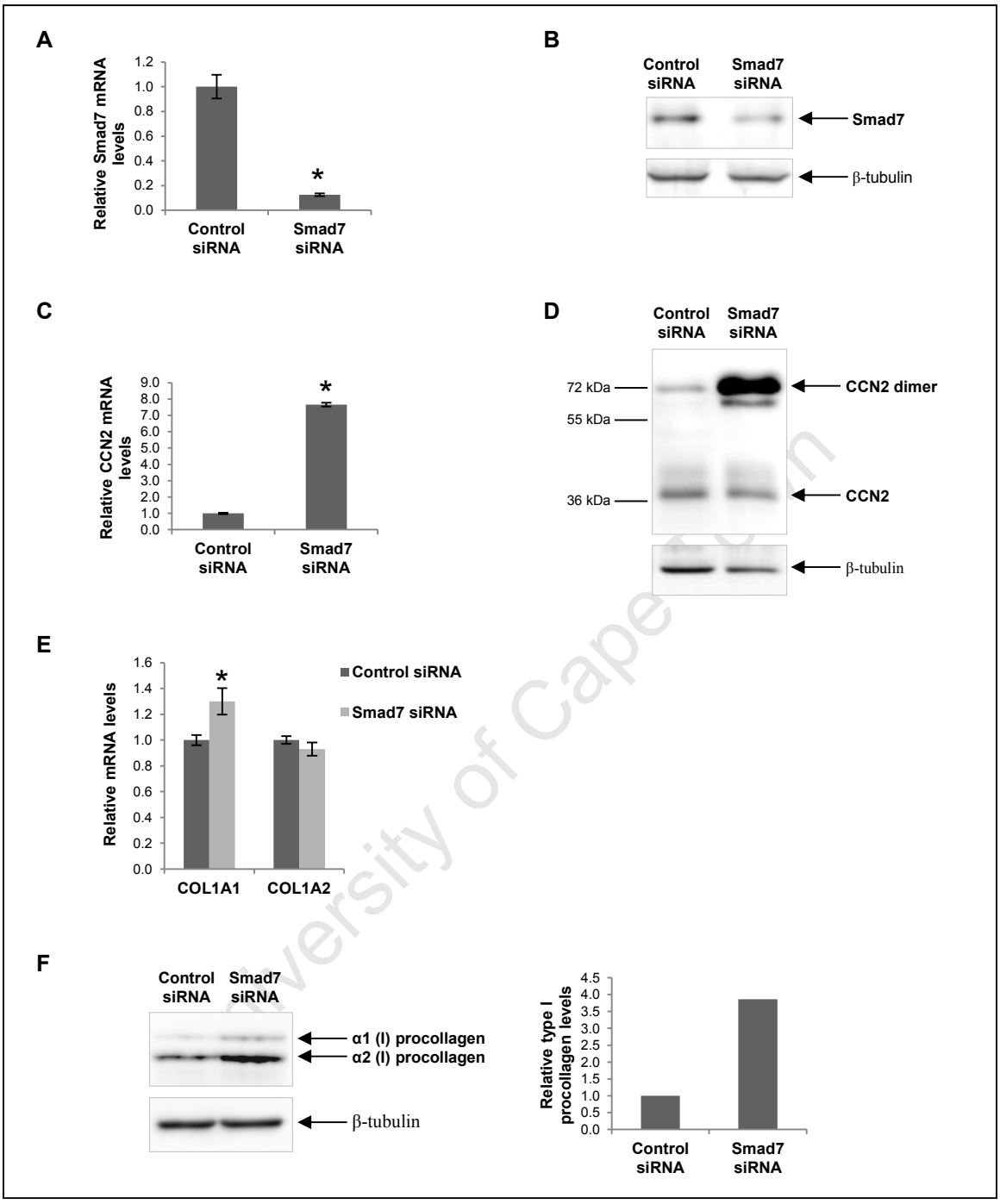

FIGURE 4.8 The effect of Smad7 inhibition on CCN2 and type I collagen gene expression in fibroblasts. CCD-1068SK fibroblasts were transfected with 80 nm Smad7 siRNA and incubated for 48 hours (section 6.16). RNA and protein was isolated from fibroblasts for further analysis. (A) Quantitative real-time PCR analysis was performed using RNA isolated from Smad7 siRNA-transfected CCD-1068SK fibroblasts and compared to levels in fibroblasts transfected with control siRNA. (B) Immunoblotting analysis of Smad7 protein in CCD-1068SK fibroblasts transfected with Smad7 siRNA. (C) Relative CCN2 mRNA levels in Smad7-inhibited CCD-1068SK fibroblasts. (D) CCN2 protein levels in Smad7-inhibited fibroblasts. The CCN2 antibody detected a band at 36-38kDa and 72 kDa, with a minor band detected just below 72 kDa. (E) Relative COL1A1 and COL1A2 mRNA levels in Smad7-inhibited CCD-1068SK fibroblasts. (F) The effect of silencing fibroblast Smad7 expression on α1(I) and α2(I) procollagen levels. The graphs show the mean ± SD from representative experiments (*$p \leq 0.05$, $n=3$).

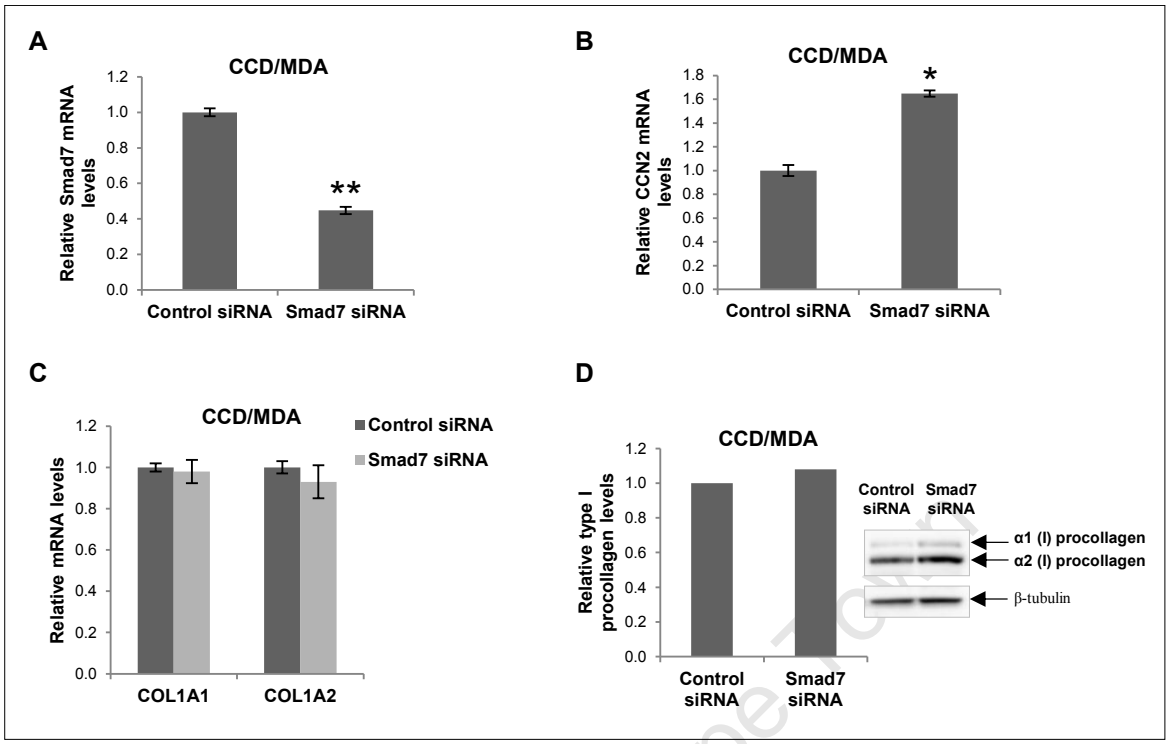

FIGURE 4.9 The effect of tumour cells on CCN2 and type I collagen gene expression in Smad7 inhibited fibroblasts during direct co-cultures. CCD-1068SK fibroblasts were transfected with 80 nm Smad7 siRNA and directly co-cultured with MDA-MB-231 tumour cells for 48 hours. As a control, fibroblasts were transfected with control siRNA before co-culture with tumour cells. RNA was isolated from co-cultured cells and used to determine relative Smad7 (A) and CCN2 (B) mRNA levels. (C) The effect of inhibiting Smad7 on relative COL1A1 and COL1A2 mRNA levels was determined in fibroblast/tumour co-cultures. (D) Immunoblotting analysis was performed on Smad7-inhibited fibroblast/tumour co-cultures, using a type I collagen antibody. The graphs show the mean ± SD from representative experiments (*$p \leq 0.05$, ** $p \leq 0.001$; $n=3$). Abbreviations: CCD, CCD-1068SK; CCD/MDA, directly co-cultured CCD-1068SK/MDA-MB-231 cells.

4.3 DISCUSSION

Gene expression analysis, performed to examine the differential expression of adhesion and ECM genes, revealed that close contact with MDA-MB-231 tumour cells resulted in the down-regulation of type I collagen and CCN2 gene expression, and an up-regulation in MMP-1 gene expression in CCD-1068SK fibroblasts. This suggests that MDA-MB-231 tumour cells cause the balance of ECM synthesis/degradation to be disturbed by decreasing the production of profibrotic type I collagen and CCN2 in neighbouring fibroblasts and concurrently causing an increase in the expression of a metalloproteinase that degrades type I collagen. Previous studies performed on highly invasive melanomas have shown that destabilization and degradation of the type I collagen matrix allows melanoma cells to evade the growth arrest and apoptosis that these cells would normally undergo in the presence of type I collagen matrix (van Kempen et al. 2005). Inhibiting MMP expression in MDA-MB-231 cells was also shown to inhibit the migration of these tumour cells through a bone marrow fibroblast monolayer (Saad et al. 2000). The results obtained in these studies suggest that the decreased type I collagen matrix production and increased MMP expression observed in co-cultured CCD-1068SK fibroblasts could facilitate MDA-MB-231 tumour cell invasion through the ECM.

Elevated expression of CCN2 has been associated with a number of fibrotic diseases (Leask et al. 2002; Liu et al. 2011; Mason 2009; Ponticos et al. 2009; Shi-wen et al. 2000; Uchio et al. 2004). However, the role of CCN2 in cancer is still controversial, with both positive and negative associations between tumour growth and CCN2 expression being observed (Chu et al. 2008; Cicha & Goppelt-Struebe 2009). This may suggest that CCN2 acts in a context-dependent manner in cancers (Oliver et al. 2010). In a study by Jiang et al. (2004) significantly lower than normal CCN2 mRNA and protein levels were detected in breast tumour samples and this was correlated with poor prognosis, with increased risk of metastasis and higher mortality rates. In our co-culture system, CCN2 mRNA and protein levels were decreased only in CCD-1068SK fibroblasts in close proximity to MDA-MB-231 tumour cells, with no change in CCN2 levels in MDA-MB-231 tumour cells. This suggests that the decrease in CCN2 gene expression observed in breast tumour tissue could be specifically localized to the stromal fibroblasts.

Existing evidence suggests that CCN2 facilitates TGFβ-induced type I collagen gene expression and that inhibition of CCN2 gene expression leads to a decrease in type I collagen protein and mRNA levels in normal skin fibroblasts (Duncan et al. 1999; Quan et al. 2009; Shi-wen et al. 2000; Shi-wen et al. 2006). In our study, silencing CCN2 expression in CCD-1068SK fibroblasts also led to a decrease in α1(I) and α2(I) procollagen mRNA and protein levels, although the effect on COL1A1 basal transcription was more pronounced than the effect on COL1A2 transcription. These results suggest that the tumour-mediated down-regulation of type I collagen gene expression observed in co-cultured fibroblasts could be a consequence of the decrease in CCN2 gene expression also observed in these fibroblasts.

Our investigations also identified Smad7 as a putative regulator of both CCN2 and type I collagen gene expression in fibroblasts, since Smad7 mRNA and protein levels were increased in CCD-1068SK fibroblasts after co-culture with tumour cells. This protein is known to act as a negative feedback regulator of TGFβ/Smad signalling (Gersdorff et al. 2000), but may also be induced by other factors such as TNFα/NFκB, EGF and IFNγ/Stat signalling (Dooley et al. 2006; Ulloa et al. 1999; Zhang et al. 2007b). Previously in this study we showed that MDA-MB-231 tumour cell co-culture caused the JAK/Stat1 signalling pathway to be activated in neighbouring CCD-1068SK fibroblasts, and it is therefore possible that activated JAK/Stat signalling could stimulate Smad7 gene expression. Although IFNγ mediated JAK/Stat signalling was previously shown to have no effect on Smad7 gene expression in fibroblasts specifically (Ghosh et al. 2001), our co-culture system involves JAK/Stat1 signalling via type I IFNs, and the mechanism of Smad7 regulation may therefore differ from that involved in IFNγ induction.

If the overexpression of Smad7 observed in CCD-1068SK fibroblasts during tumour co-culture did indeed occur via a TGFβ-independent mechanism, there are a number of implications in terms of the effect on tumour progression. Since Smad7 negatively regulates the cellular response to TGFβ, overexpression of Smad7 may result in unresponsiveness of fibroblasts in the tumour milieu to TGFβ signals. Recent evidence

indicates that fibroblasts unable to respond to TGFβ may facilitate tumour growth (Bhowmick et al. 2004). By transplanting fibroblasts lacking the TGF-β receptor into mice together with mammary carcinoma cells, the aggressiveness and metastatic ability of the resulting tumours was increased when compared to that observed in tumour cells transplanted together with normal fibroblasts. The altered fibroblasts produced TGFα and hepatocyte growth factor (HGF) which resulted in accelerated tumour cell growth. Since TGFβ also usually suppresses destructive immune and inflammatory responses (Kunz-Schughart & Knuechel 2002b; Massague 2008), preventing the action of this tumour suppressor in breast cancer could result in tumour-promoting inflammatory conditions (Bierie & Moses 2006; Mantovani et al. 2008).

The important role of Smad7 in regulating basal levels of CCN2 gene expression was revealed as a result of Smad7 inhibition studies in CCD-1068SK fibroblasts, with decreased Smad7 expression resulting in a significant increase in CCN2 mRNA and protein levels. To our knowledge, this is the first study to examine the effect of Smad7 inhibition on CCN2 gene expression. Previous studies have shown that overexpression of Smad7 reduces TGFβ-stimulated CCN2 gene expression, but has no effect on the basal expression of CCN2 (Holmes et al. 2001). However, since we have shown earlier that CCD-1068SK fibroblasts produce TGFβ in monocultures (3.2.1), it is possible that Smad7 plays a role in negatively regulating autocrine TGFβ signalling in these fibroblasts and that removal of this regulation results in increased CCN2 gene transcription. Smad7 inhibition in CCD-1068SK fibroblasts also caused an increase in type I procollagen levels, although a less pronounced effect was observed at an mRNA level. The reason for this is not known, but may indicate that Smad7 regulates basal type I collagen gene expression by decreasing COL1A1 and COL1A2 mRNA stability rather than controlling gene expression at a promoter level. Inhibition of Smad7 gene expression in co-cultured fibroblasts resulted in a significant increase in CCN2 gene expression compared to that observed in control co-cultures. However, the level of reversion in CCN2 mRNA levels observed in Smad7-inhibited co-cultures may not account for the total negative effect of tumour cells on CCN2 gene expression, since there was originally more than a 5-fold decrease in CCN2 mRNA levels in fibroblasts as a result of tumour cell co-culture. Smad7 inhibition also did not

revert the negative regulatory effect of tumour cells on type I collagen gene expression. These results suggest that Smad7 may at least partially be involved in negatively regulating CCN2 gene expression during tumour co-culture, but that other factors may also be involved in tumour-mediated suppression of both CCN2 and type I collagen gene expression.

In this study we suggest a potential role for CCN2 in the tumour-mediated regulation of type I collagen, with at least partial regulation of CCN2 by tumour cells occurring via a Smad7-dependent mechanism. We also show that invasive MDA-MB-231 tumour cells cause normal fibroblasts to decrease their expression of profibrotic genes while increasing collagenase production when there is close contact between tumour cells and fibroblasts. This tumour-mediated initiation of matrix degradation by neighbouring normal fibroblasts could allow the tumour cells to escape the inhibitory effects of the matrix and promote further tumour migration and invasion.

CHAPTER FIVE

CONCLUSION

It has recently been shown that genetic mutations are not the only factors that play a role in the progression of the transformed epithelial cells to an invasive tumour cells, but that continuous communication with the surrounding stroma may also facilitate tumour development. If tumours progress to the invasive stage, the basement membrane which usually separates the tumour cells from the fibroblasts in degraded, allowing tumour cells to invade into the surrounding stroma where they come into close contact with stromal fibroblasts. Since these fibroblasts are the main producers of the components making up the ECM, close interactions with tumour cells could influence ECM production by fibroblasts with further consequences for tumour migration and invasion.

Type I collagen is the main constituent of the ECM, and previous studies have shown that type I collagen gene expression is decreased in stage II and stage III breast tumours when compared to levels in normal tissue (Fenhalls et al. 1999). Since further *in vivo* studies showed that breast tumour cells had a negative regulatory effect on type I collagen gene expression in neighbouring fibroblasts, we aimed to determine the mechanisms involved in this regulation. The role of the MDA-MB-231 breast tumour cell line in regulating type I collagen gene expression in normal breast skin (CCD-1068SK) and lung (WI38) fibroblasts was determined using direct and indirect co-culturing systems. The role of tumour-secreted factors and fibroblast signalling pathways in mediating the effects on type I collagen gene expression in normal fibroblasts was also further investigated. Microarray analysis was performed to determine the effect of close contact with tumour cells on the expression of cell adhesion and ECM molecules in fibroblasts.

The results of our study show that MDA-MB-231 tumour cells require close contact with normal CCD-1068SK and WI38 fibroblasts to negatively regulate type I collagen gene expression. This negative regulation is specifically a result of a factor produced by the tumour cells, and is not observed in co-cultures with non-tumourigenic MCF12A epithelial cells. The expression of both the COL1A1 and the COL1A2 genes were down-regulated,

with specific involvement of the -375/+54 region of the COL1A2 promoter, suggesting that tumour cells regulate type I collagen gene expression at a transcriptional level in co-cultured fibroblasts. Type I collagen mRNA levels were decreased as early as four hours after co-culture and, since tumour-mediated repression of type I collagen mRNA and protein levels was still observed six days after co-culture, the tumour-produced factor responsible for this regulation was not only transiently expressed during co-cultures.

Tumour-mediated regulation of type I collagen gene expression in fibroblasts may occur via secreted factors and/or cell surface adhesion molecules. Previous studies have shown that factors (TNFα, IFNγ and IL-1) secreted by keratinocyte and T-cells can negatively regulate type I collagen gene expression in fibroblasts, but that close contact between cells was required. Our investigations revealed that TNFα, IFNγ and IL-1 were not secreted during MDA-MB-231/CCD-1068SK co-cultures, but that the expression of IFNα and IFNβ was significantly increased under these conditions. These type I IFNs appeared to mediate their effect through the JAK/Stat signalling pathway, with increased gene expression and activation of Stat1 observed in both fibroblasts and tumour cells after co-culture. Since the increase in Stat1 levels observed in the fibroblasts was shown to occur only in response to factors secreted during direct fibroblast/tumour co-cultures, we suggest that a tumour cell factor(s) triggers fibroblasts to produce type I IFNs during direct co-culture and that the secreted type I IFNs signal in both an autocrine and paracrine manner, resulting in JAK/Stat activation in both cell types as well as a further increase in type I IFN gene expression. Stat1 gene expression has previously been shown to be specifically up-regulated at the tumour-stroma border in certain breast tumours (Buess et al. 2007) and our results suggest that it is specifically the close association of tumour cells with stromal fibroblasts that leads to increased Stat1 gene expression and activation. The increase in Stat1 gene expression has also been inversely correlated with poor patient outcome (Buess et al. 2007) and since close contact between CCD-1068SK fibroblasts and MDA-MB-231 tumour cells also increases type I IFN expression by both cell types, the resulting recruitment of immune and inflammatory cells to the tumour could result in an inflammatory condition which may facilitate further tumour cell invasion.

Further investigation of the role of Stat1 in type I collagen gene expression showed that the negative regulatory effect of tumour cells on type I collagen gene expression could not be reversed by inhibiting JAK activation and Stat1 gene expression. However, indirect co-culture results did suggest that type I collagen gene expression was not affected by factors secreted as a result of fibroblast/tumour co-culture. Type I collagen down-regulation therefore occurs as a result of a tumour cell factor(s) that only has an effect on fibroblasts in close contact with these tumour cells.

Since MAPK signalling pathways are often deregulated in tumours, the role of the MEK/ERK, JNK and p38 MAPK signalling pathways in tumour-mediated negative regulation of type I collagen gene expression was also investigated. Inhibition studies performed on co-cultured cells using U0126 and SP600125 showed that MEK/ERK and JNK signalling pathways were not involved. However, results of studies using SB203580 suggests that the p38 MAPK signalling pathway may at least be partially involved in regulating type I collagen gene expression in response to tumour cell co-culture.

Since none of the secreted factors known to negatively regulate type I collagen gene expression were produce during tumour/fibroblast co-cultures, the role of cell adhesion and ECM molecules in type I collagen regulation were investigated. MMP-1 (collagenase-1) was shown to be overexpressed in CCD-1068SK fibroblast as a result of tumour co-culture, while CCN2 gene expression was down-regulated. CCN2 is a matricellular protein that is thought to play a role in regulating type I collagen gene expression in fibroblasts and is regarded as a reliable marker of fibrosis (Shi-wen et al. 2000). Silencing CCN2 gene expression in CCD-1068SK fibroblasts led to a decrease in type I collagen gene expression, which suggests that the tumour-mediated down-regulation of CCN2 levels observed in co-cultured fibroblasts could be resulting in the decrease in type I collagen gene expression.

The role of Smad7 as a possible tumour cell-induced negative regulator of CCN2 and type I collagen gene expression was also investigated. Protein and mRNA levels of this negative regulator of the Smad signalling pathway were increased in CCD-1068SK

fibroblasts as a result of direct co-culture with tumour cells. Increased Smad7 gene expression is known to suppress TGFβ-stimulated Smad signalling, and fibroblasts unable to respond to TGFβ appear to facilitate tumour growth (Bhowmick et al. 2004). Therefore, any down-stream effects of inhibiting Smad pathway activation may be involved in facilitating tumour growth. Smad7 inhibition in fibroblasts resulted in a down-regulation of basal CCN2 gene expression and was also able to at least partially reverse the tumour-suppressive effect on CCN2 gene expression. Basal expression of type I procollagen was also increased as a result of Smad7 inhibition in fibroblasts. However, no effect on type I collagen gene expression was observed in Smad7-inhibited fibroblasts co-cultured with tumour cells. These results suggest that the tumour-stimulated increase in Smad7 gene expression plays a role in negatively regulating CCN2 gene expression, possibly by inhibiting Smad signalling, but that other signalling pathways and/or transcription factors may also contribute to tumour-mediated suppression of CCN2 and type I collagen gene expression.

Since tumour cells need close contact with fibroblasts to suppress type I collagen gene expression, cell-membrane associated adhesion molecule may also be involved in this regulation. Although our microarray analysis did not reveal any changes in the expression of integrins or other cell adhesion molecules, tumour-mediated regulation might not affect gene expression but could rather involve conformational changes to the integrin subunits, redistribution of adhesion molecules on the cell surface and/or association of cell adhesion molecules with growth factor or cytokine receptors (Comoglio et al. 2003; Danen & Sonnenberg 2003; Ivaska & Heino 2011). Recent studies have shown that TGFβ-mediated up-regulation of both CCN2 and type I collagen in fibroblasts requires activation of Alk1/Smad1 and ERK1/2 signalling (Pannu et al. 2007) and that the association of CCN2 with β_3 integrin is required for TGFβ-mediated Smad1 phosphorylation (Nakerakanti et al. 2011). Silencing Smad1 gene expression resulted in a decrease in the expression of both TGFβ-stimulated CCN2 and type I collagen gene expression as well as basal type I collagen expression (Pannu et al. 2007). CCN2-deficient embryonic fibroblasts have also shown an impaired ability to induce adhesive signals and type I collagen gene expression in response to TGFβ stimulation in a Smad-independent manner involving FAK (focal

adhesion kinase) and Src kinase (Nakerakanti et al. 2011; Shi-wen et al. 2006). Further studies would therefore need to be undertaken to examine the role of cell adhesion molecules and downstream signalling pathways in tumour-fibroblast interactions and, more specifically, their role in regulating CCN2 and type I collagen gene expression by tumour cells.

The results obtained in this study suggest that tumour cells are able to down-regulate the expression of profibrotic genes such as type I collagen and CCN2, while up-regulating the expression of collagenases such as MMP-1 in neighbouring fibroblasts when in close contact. Since this type of close contact between tumour cells and fibroblasts is only possible in the later stages of breast cancer progression, the resulting decrease in fibroblast-mediated production of the surrounding extracellular matrix could facilitate further tumour invasion and metastasis. The contribution we have made to better understanding the communication occurring between tumour cells and fibroblasts during tumour invasion, including how this affects ECM synthesis and degradation, may therefore assist in the identification of key mediators of tumour invasion with implications for the development of novel therapeutic drug targets.

CHAPTER SIX

MATERIALS AND METHODS

6.1 CELL CULTURE

CCD-1068SK breast fibroblasts, WI38 lung fibroblasts, MDA-MB-231 breast tumour cells and MCF12A non-tumorigenic epithelial cells were purchased from the American Type Culture Collection (ATCC). All cells, apart from MCF12As, were grown in complete medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 100 μg/ml streptomycin and 100 U penicillin. MCF12A cells were grown in a 1:1 mixture of DMEM and Ham's F12 medium containing 5 % FCS, 20 ng/ml human epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin and 500 ng/ml hydrocortisone.

6.2 DIRECT CO-CULTURE

Fibroblasts were mixed with an equal number of MDA-MB-231 tumour cells and seeded into dishes or plates. As controls, fibroblast were co-cultured with an equal numbers of MCF12A cells and each cell type was cultured on its own. Cells were allowed to settle in complete medium for at least 12 hours. Cells were then washed twice with 1 X PBS and incubated in serum-free DMEM containing 50 μg/ml ascorbic acid for a further 48 hours, unless otherwise indicated. All experiments were performed in triplicate and experiments were repeated at least once.

6.3 INDIRECT CO-CULTURE

CCD-1068SK or WI38 fibroblasts were seeded into 6-well plates and on transwell inserts (NUNC) in separate 6-well plates. An equal number of MDA-MB-231 or MCF12A cells were also seeded on transwell inserts in separate 6-well plates. The pore size of the insert membrane was 0.2 μm. In certain cases mixed MDA-MB-231/CCD-1068SK cells were also seeded in transwell inserts. Cells were allowed to settle in complete medium for at least 12 hours before inserts were transferred into the 6-well plates containing fibroblasts. Medium was removed, cells were washed twice with 1 X PBS and serum-free DMEM containing 50 μg/ml ascorbic acid was added. Cultures were incubated for a further 48

hours. All experiments were performed in triplicate and experiments were repeated at least once.

6.4 FLUORESCENT CELL LABELLING

Fibroblasts were labelled with PKH67 green fluorescent dye (Sigma) according to the manufacturer's instructions. Cells were removed from dishes using trypsin/EDTA and pooled. Approximately 2 X 10^7 cells were placed in a conical polypropylene tube and washed once with serum-free medium. The supernatant was removed after centrifugation and cells were resuspended in 1ml of Diluent C. A 2x dilution of PKH67 dye (4 X 10^{-6} molar) was made up in 1ml of Diluent C and added to the cell suspension. Cells were mixed well and incubated at room temperature for 5 minutes. The reaction was stopped by adding an equal volume of serum and incubated for a further minute. An equal volume of complete medium was added and samples were centrifuged at 400 x g for 10 minutes. The pellet was resuspended in 10 ml of complete medium, transferred to a new tube and washed at least three times before seeding 1.4 X 10^6 cells per 150mm dish. An equal number of MDA-MB-231 cells were added, and incubated as for direct co-cultures. CCD-1068SK and MDA-MB-231 monocultures were used as controls.

6.5 FLUORESCENCE-ACTIVATED CELL SORTING

Co-cultured cells were trypsinized, centrifuged and resuspended in DMEM with 2 % FCS. Cell types were sorted based on green fluorescence using the BD FACS VANTAGE. The separated cells were collected in tubes containing DMEM with 2% FCS and used for further RNA and protein analysis.

6.6 RNA ISOLATION

Qiazol (Qiagen) was used to extract RNA from cells according to the manufacturer's instructions. After removing the medium, cells were washed twice with ice-cold 1 X PBS. All further steps were performed on ice. Qiazol was added to cells and the lysate was resuspended and transferred to 1.5 ml tubes. Chloroform was added to each tube, the suspension was mixed vigorously and then centrifuged at 12 000 x g for 15 minutes at 4 °C. The aqueous phase containing the RNA was transferred to new tubes and isopropyl alcohol was added. RNA was precipitated overnight at -20 °C and pellets were collected by centrifugation at 12 000 x g for 30 minutes at 4 °C. The pellet was washed once with

75 % EtOH and air dried for 5 to 10 minutes. The RNA was dissolved in RNase-free water and the concentration was determined by means of spectrometry at 260 nm. RNA quality was checked by running 1 µg of sample on a 2.5 % MOPS agarose gel and visualizing ethidium bromide under UV light. Samples were stored at -80 °C.

6.7 REVERSE TRANSCRIPTION

The ImProm^{TM}-II Reverse Transcription System (Promega) was used to synthesize cDNA according to the manufacturer's protocol. Firstly, 1 µg of RNA was made up to a total volume of 7 µl and 1 µl of Oligo(dT)$_{15}$ (500ng) was added. Samples were incubated at 70 °C for 10 minutes and then on ice for 5 minutes. The reverse transcription reaction mix was made up by adding together 1 µl of ImProm-II^{TM} Reverse Transcriptase, 4 µl of ImProm-II^{TM} 5X Reaction Buffer, 2.4 µl of $MgCl_2$ (3 mM) , 1 µl dNTPs (0.5mM of each dNTP), 0.5ul RNasin® ribonuclease inhibitor (20U) and RNase-free water to a total volume of 12 µl. The reverse transcription reaction mix was then added to the RNA/Oligo(dT)$_{15}$ mix and incubated for 5 minutes at room temperature to allow primer annealing, and then at 42 °C for 90 minutes to allow reverse transcription. The reverse transcriptase was inactivated by incubation of samples at 70 °C for 10 minutes. Samples were stored at –80 °C until further use.

6.8 REAL-TIME PCR

For each real-time PCR reaction 2 µl of cDNA was added to 12.5 µl KAPA SYPR green, 1 µl of forward and reverse primer and 8.5 µl of water. Real-time PCR reactions were performed on a MiniOpticon^{TM} Real-Time PCR Detection System (BIO-RAD) or the Roche LightCycler® 480. GAPDH was used to normalize results and the $2^{-\Delta\Delta C}$T method (Livak & Schmittgen 2001) was used to analyse results.

Table 6.1 Real-time PCR primer information.

Gene name	Primers	Annealing temperature	Product size	Reference
GAPDH	F: 5'-GGCTCTCCAGAACATCATCC-3' R: 5'-GCCTGCTTCACCACCTTC-3'	60 ºC	192 bp	-
COL1A1	F: 5'-CAGCCGCTTCACCTACAGC-3' R: 5'-TTTTGTATTCAATCAGTGTCTTGCC	60 ºC	83 bp	Martin et al., 2001
COL1A2	F: 5'-GATTGAGACCCTTCTTACTCCTGAA-3' R: 5'- GGGTGGCTGAGTCTCAAGTCA-3'	60 ºC	78 bp	Sengupta et al., 2003
IFNα	F: 5'- CCTCGCCCTTTGCTTTACTG-3' R: 5'-GCCCAGAGAGCAGCTTGACT-3'	65 ºC	65 bp	Buess et al., 2007
IFNβ1	F: 5'-ACCTCCGAAACTGAAGATCTCCTA-3' R: 5'- TGCT- GGTTGAAGAATGCTTGA-3'	65 ºC	74 bp	Buess et al., 2007
Stat1	F: 5'- ATGGCAGTCTGGCGGCTGAATT-3' R: 5'- CCAAACCAGGCTGGCACAATTG-3'	60 ºC	256 bp	-
CCN2	F: 5'-GTTTGGCCCAGACCCAACT-3' R: 5'-GTGCAGCCAGAAAGCTCAAA-3'	60 ºC	650 bp	-
MMP1	F: 5'-ATCCACTCCCCATTTCACAA-3' R: 5'-TCCTGCAGTTGAACCAGCTA-3'	60 ºC	867 bp	-
Smad7	F: 5'-CCAGATAATTCGTTCCCCCTGT-3' R: 5'-CCTTAGCCGACTCTGCGAACTA-3'	60 ºC	137 bp	Koinuma et al 2003

6.9 PROTEIN ISOLATION

After removing the medium, cells were washed twice with 1 X PBS and an appropriate volume of 1 X RIPA buffer (Cell Signaling) containing 1 X Protease inhibitor (Roche) and 1 X phosphatase inhibitor (Roche) was added. Cells were scraped off the dish, and the lysate was transferred to 1.5ml tubes. Samples were sonicated for 10 seconds and centrifuged at 10 000 rpm for 10 minutes at 4 °C to remove cell debris. The supernatant was transferred to a new tube and stored at -20 °C.

6.10 PROTEIN QUANTIFICATION

The BCA™ Protein assay kit (Pierce) was used to quantify protein levels. Protein sample (2.5 µl) was added to 22.5µl of water in a 96-well plate. BSA Concentration Standards (2 000 µg/ml to 0 µg/ml) were added to separate wells. Two hundred microliters of BCA Working Reagent (50:1 reagent A:reagent B) was then added to samples and concentration

96-well plate reader. A standard curve of BSA standards was drawn up and used to determine the protein concentration of the unknown samples.

6.11 SDS-PAGE AND IMMUNOBLOTTING ANALYSIS

Between 6 and 15 % SDS-Page separating gels were made with a 4 % stacking gel. Approximately 20 to 30ug of protein was denatured by incubation at 95 °C for 10 minutes in protein loading buffer and then loaded on SDS-Page gels. Gels were run at 160 V for 90 minutes and then transferred to nitrocellulose membranes for a further 90 minutes at 100 V. Membranes were blocked in 5 % milk for 1 hour. After 3 washes with TBST, primary antibody resuspended in the appropriate buffer (table 6.2) was added to membranes and incubated at 4 °C, overnight with shaking. Three further TBST washes were performed before incubating membranes in secondary antibody in appropriate buffer (table 6.3) for 1 hour at room temperature. After 5 washes with TBST, membranes were incubated with LumiGlo Reserve (KPL) for one minute. Chemiluminscence was detected on X-ray film or using a UVP GelDoc-It® TS imaging system.

Table 6.2 Primary antibody information for immunoblotting.

Primary antibody	WB conditions	Product size (kDa)	Company
Anti-Type I collagen	1:1000 in 5% milk	130-250	Southern biotech
B-tubulin	1:1000 in 5% milk	55	Santa Cruz
p-STATI (Tyr 701)	1:1000 in 2.5% BSA	84 & 91	Cell Signaling
STATI	1:1000 in 1% BSA	84 & 91	Cell Signaling
cJun	1:1000 in 1% BSA	39	Santa Cruz
p-cJun (Ser63/73)	1:1000 in 1% BSA	46 & 54	Santa Cruz
p-Erk 1,2	1:500 in 1% BSA	44 & 42	Cell Signaling
Erk2	1:1000 in 1% milk	42	Santa Cruz
p-p38 (Thr 180/Tyr 182)	1:1000 in TBST	43	Cell Signaling
p38	1:5000 in TBST	38	Sigma
CCN2	1:1000 in 5% BSA	36-38	Santa Cruz
Smad7	1:500 in 5% BSA	46	Santa Cruz

Table 6.3 Secondary antibody information for immunoblotting.

Secondary antibody	WB conditions	Company
Donkey anti-goat	1:3000 in 5% milk	Southern biotech
Goat anti-rabbit	1:5000 in 5% milk	Bio-rad
Goat anti-mouse	1:1000 in 2.5% milk	Bio-rad

6.12 ANALYSING THE INCORPORATION OF [^{3}H]-PROLINE INTO SECRETED α1(I) AND α2(I) PROCOLLAGEN.

CCD-1068SK or WI38 fibroblasts were mixed with an equal number of MCF12A or MDA-MB-231 cells, seeded into 6-well plates and allowed to settle overnight. Cells were then washed twice with 1 X PBS, after which 2 ml serum-free DMEM with 20 μCi/ml [^{3}H]-proline (American Radiolabeled Chemicals Inc), 50 mg/ml ascorbic acid and 50 mg/ml β-aminopropionitrile was added to each well and incubated for 20 hours.

Medium was removed from cells and transferred to 2ml microfuge tubes. Acetic acid was added to a final concentration of 0.5M. Medium proteins were then digested with 100 μg/ml pepsin for 4 h at 20 °C, with rotation. Digested medium was transferred to dialysis tubing and dialyzed overnight against 50mM Tris, pH 7.5, with one buffer change after 2 hours. Medium was transferred back into microfuge tubes and precipitated with TCA overnight at 4 °C. The samples were centrifuged at 11 000 rpm for 15 minutes, washed twice with acetone, air-dried and dissolved in 40 μl of SDS-Page loading buffer. Ten microliters of sample was added to 1 ml of scintillation fluid and radioactivity was determined using a Packard scintillation counter. An equal volume of each sample was heat-denatured at 95 °C for 5 minutes and run on a 7% SDS-Page gel (with 4 % stacking gel) for 80 minutes at 180 V. The gel was soaked in 1M sodium salicylate for 1 hour and then washed in distilled water for another hour. The gel was then placed on 3mm whatmann paper, covered with saran wrap and vacuum dried at 70 °C for 2 hours. The dried gel was placed in a cassette and exposed to film for 7 days at -80 °C, after which it was developed and fixed.

6.13 PLASMID TRANSFECTIONS

WI38 or CCD-1068SK fibroblasts were seeded into 10cm dishes and allowed to settle overnight to obtain a final confluence of 60 %. The next morning, the transfection mix was made up according to the manufacturer's instructions (Polyplus transfection) by diluting 10ug of the plasmid of interest in 500 μl of 150mM NaCl. One microgram of CMV-βgal plasmid, containing a β-galactosidase gene driven by a CMV promoter, was added as an internal control. In a separate tube, 20 μl of jetPEI^{TM} transfection reagent was added to 500 μl NaCl. The jetPEI^{TM} solution was then added to the plasmid solution,

vortexed briefly and incubated at room temperature for 30 min. Medium was changed on the WI38 cells, the jetPEI™/plasmid mixture was added drop-wise to the cell medium, and cells were incubated for 24 hours. After 24 hours, the transfected fibroblasts were trypsinized and counted. The cells were split into 9 wells of 6-well plates and an equal number of MDA-MB-231 or MCF12A cells (if applicable) were added to three wells each. Co-cultures were incubated for 12 hours in complete medium and a further 12 hours in serum-free medium before undergoing further analysis.

6.14 LUCIFERASE ASSAYS

The Promega Luciferase Assays System was used to determine luciferase levels. Briefly, cell were washed with 1X PBS, actively lysed in 1X passive lysis buffer and the supernatant was transferred to white, 96-well plates for analysis. Luciferase Assay Reagent II (LARII) was added to the lysate and luciferase levels were quantified using a luminometer. To determine β-galactosidase levels for normalization, Beta-Glo® was added to a separate aliquot of the lysate, incubated for 30 minutes at room temperature and analysed with a luminometer. Luciferase and β-galactosidase values were corrected by subtracting the background values obtained for each cell type (untransfected). The corrected luciferase value for each sample was subtracted from the corrected β-galactosidase value to obtain relative luciferase activity.

6.15 CYTOKINE ELISA

The Multi-Analyte Profiler ELISarray kit (SABioscience) was used to determine the levels of the following 12 cytokines in culture medium: IL-1b, IL-4, IL-6, IL-10, IL-12, IL-17A, IFNγ, TNFα, TGF-β1, MCP1, MIP-1a, and MIP-1b. Experiments were performed according to the manufacturer's instructions. Briefly, 50 µl of assay buffer were added into each well of the ELISarray plate. A further 50 µl of sample (culture medium) was added to the appropriate wells. Antigen standards were made up and added to the appropriate well in row A. Medium was added to the last row as a negative control. After a two hour incubation the plate was washed 3 times with the provided wash buffer. Detection Antibody Solution (100 µl) was added to all wells and the plate was incubated for 1 hour. After 3 washes, 100 µl of Avidin-HRP was added and incubated for 30 minutes. The plate was washed 4 times and 100 µl of Development Solution was added.

The plate was incubated in the dark for 15 minutes and 100 µl of Stop Solution was added to all wells. The plate was read at OD 450 and OD 570 within 30 minutes of adding Stop Solution. Values were normalized by subtracting the OD570 values from OD450 values and dividing this value by total cell number.

6.16 CO-CULTURES WITH SIGNALLING PATHWAY INHIBITORS

For co-cultures, CCD-1068SK fibroblasts were mixed with an equal number of MCF12A or MDA cells or seeded alone in 6-well plates and allowed to settle overnight. Cells were washed with 1X PBS and serum-free medium was added to cells and incubated for 24h. Fresh serum-free medium containing the appropriate volume of inhibitor was added to cells and incubated for a further 24 hours. U0126, SP600125 and SB203580 were obtained from Merck, while JAK inhibitor I was a Santa Cruz product. Control cells were incubated with 0.1 % DMSO in serum-free medium. RNA and protein was then isolated and used for further analysis as previously described.

6.17 siRNA TRANSFECTIONS

CCD-1068SK or WI38 fibroblasts were plated in 6-well plates and allowed to settle overnight, to reach a final confluence of 50%. Transfectin lipid reagent (Bio-Rad) was added to the appropriate volume of siRNA in DMEM and incubated at room temperature for 20 minutes before being added dropwise to cell medium. Stat1, CCN2 and Smad7 siRNA pools were all Dharmacon products (Thermo Scientific). Cells were then incubated overnight with the transfection mix. The next day, medium was changed and an equal number of MDA-MB-231 cells were added and allowed to settle overnight. Serum-free medium was added to co-cultured cells and incubated for a further 24 hours before continuing with RNA and protein extraction as previously described.

6.18 OLIGO GEarray® HUMAN EXTRACELLULAR AND ADHESION MOLECULES MICROARRAY ANALYSIS.

CCD-1068SK fibroblasts were fluorescently labelled, directly co-cultured with an equal number of MDA-MB-231 for 48 hours and separated by means of FACS, as previously described (see 6.4 & 6.5). RNA was extracted from CCD-1068SK fibroblast and the RNeasy® MinElute™ Cleanup Kit (Qiagen) was used to purify the RNA (according to the manufacturer's protocol). The eluted RNA was quantified and 3 µg of each RNA sample

was used for cDNA and cRNA synthesis, using the TrueLabeling-AMP™ 2.0 kit (SABiosciences). cRNA was purified using the ArrayGrade™ cRNA Cleanup Kit (SABiosciences) and quantified.

The Oligo GEArrays® nylon membranes were pre-wet with deionized water. Prewarmed GEAhyb Hybridization Solution was added and the membranes were prehybridized at 60 °C, with rotation, in a hybridization oven for more than one hour. Approximately 6 µg of cRNA was added to 0.75 ml of prewarmed GEAhyb Hybridization Solution and added to the hybridization tube after discarding the prehybridization solution. Membranes were hybridized at 60 °C, with continuous rotation, overnight. A low stringency wash was followed by high stringency wash of the membranes, performed at 60 °C for 15 minutes each. Tubes were then allowed to reach room temperature in the hybridization oven.

The Chemiluminescent Detection Kit (SABiosciences) was used for binding of the alkaline phosphatase-conjugated streptavidin and for detection of biotinylated cRNA probes. After adding CDP-Star chemiluminescent substrate to the membranes the array images were detected using the Syngene G:Box Chemi system. The images were uploaded onto the web-based GEArray Expression Analysis Suite for further evaluation. The background was normalized against two empty spots on each array and gene expression was normalized against ribosomal protein S27a (RPS27A) and beta actin (ACTB) gene expression.

6.19 IMMUNOBLOTTING ANALYSIS OF CCN2 IN CELL MEDIUM

CCD-1068SK fibroblasts were directly co-cultured with MDA-MB-231 tumour cells for 48 hours in serum-free medium as previously described. Medium was removed from cells and dialyzed against distilled water, overnight at 4 °C. Samples were then lyophilized overnight and resuspended in sterile water. An equal concentration of sample protein was then run on a 15% SDS-Page gel and used for immunoblotting analysis as previously described (see 6.10).

6.20 STATISTICAL ANALYSIS

All experiments were performed in triplicate and repeated at least twice. Standard deviations (SD) were calculated from triplicate samples by means of the two-tailed Student's t-test and a p-value ≤ 0.05 was regarded as statistically significant.

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